IMMUNOFLUORESCENCE WITH MYCOPLASMA HYORHINIS AND A STEROL-REQUIRING STRAIN OF MYCOPLASMA GRANULARUM

by

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A Thesis Submitted to the
Graduate Faculty in Partial Fulfillment of
The Requirements for the Degree of
MASTER OF SCIENCE

Major Subject: Veterinary Microbiology

Iowa State University
Of Science and Technology
Ames, Iowa

1970

TABLE OF CONTENTS

INTRODUCTION

The production of polyserositis-arthritis in young swine by a mycoplasma was reported by Switzer in 1953 (164, 165). Since this initial work several diseases of swine such as enzootic pneumonia (66, 109), uncomplicated arthritis (119, 161) and mastitis-metritis (118) have been attributed to mycoplasmas.

Mycoplasma hyorhinis and Mycoplasma granularum have been isolated from the nasal cavities of a large proportion of swine, but several mycoplasmas serologically unrelated to known swine mycoplasmas have also been isolated from swine nasal cavities (41, 152, 181). Suitable procedures such as immunofluorescence, thus need to be developed to facilitate the rapid, efficient differentiation of mycoplasmas isolated from swine.

Mycoplasma hyorhinis produces polyserositis and arthritis in swine under 6 weeks of age. The organism probably gains entrance into swine after damage to the respiratory tissue such as occurs with atrophic rhinitis and enzootic pneumonia.

Uncomplicated arthritis develops in 100-200 lb pigs infected with Mycoplasma granularum with a higher incidence in purebred, well-muscled pigs. The organism apparently invades pigs from the upper respiratory tract but the disease is not often associated with rhinitis or pneumonia. Since the organism is frequently isolated from tonsils and lymph nodes of infected pigs, the lymphatic system may be a significant avenue of spread of the organism within the pig.

Economic loss due to mycoplasmal polyserositis results from

the extended period required to market pigs. Approximately 5 to 10 per cent of market weight swine exhibit residual lesions of this disease. Less concrete evidence is available on the economic significance of arthritis due to Mycoplasma granularum. However, this organism was found to be responsible for the greatest proportion of uncomplicated arthritis in young adult pigs in the Midwest.

One of the many voids in information on the pathogenesis of mycoplasmal arthritis is that the organisms have not been visualized in tissues of infected pigs (45, 140, 143). Information of this nature would result in a clearer understanding of the pathogenesis of arthritis due to both Mycoplasma hyorhinis and Mycoplasma granularum.

Mycoplasmal arthritis of swine resembles rheumatoid arthritis of man in certain respects (45, 146). Since mycoplasmas may play a role in the etiology of rheumatoid arthritis, elucidation of the pathogenesis of swine arthritis due to mycoplasmas could possibly advance knowledge regarding the pathogenesis of that disease.

Direct immunofluorescence was used to differentiate mycoplasmas in fluid or solid media, to study their growth in cell cultures and in some cases to follow the pathogenesis of mycoplasmal infection in an animal host, but it has not been used appreciably with swine mycoplasmas. This thesis pertains to the adaptation of direct immunofluorescence for the study of Myco-

plasma hyorhinis and Mycoplasma granularum in fluid media, swine synovial cell cultures and lesions of infected pigs.

LITERATURE REVIEW

Mycoplasma Hyorhinis

History

In a series of publications by Switzer, a filterable organism originating from the nasal cavity of swine was identified as a mycoplasma, characterized and named Mycoplasma hyorhinis (160, 163, 164, 165, 166). This was the first mycoplasma found to be pathogenic for swine and although it seems that McNutt et al. (116) encountered the same organism in 1949, they did not characterize the "active agent" which had been isolated from arthritic swine joints, nor was it propagated on lifeless media. Since this initial work, a considerable amount of material on this organism has been published, including several papers of a review nature (94, 115, 137, 142, 146, 161, 162, 166, 167, 168, 169, 170).

Laboratory propagation and growth characteristics

Mycoplasma hyorhinis was initially isolated and propagated in 5 to 8 day old embryonated hens' eggs (18, 36, 41, 46, 52, 72, 97, 116, 150, 164). Inoculation of infective material into the yolk sac of chicken embryos was more successful than inoculation into the allantoic sac (97, 164), amniotic cavity (97, 184) or onto the chorio-allantoic membrane (164). Mycoplasma hyorhinis produced an erratic pattern of deaths in chicken embryos (41, 164), but some strains regularly produced a high mortality (97, 185). Titration trials with Mycoplasma

hyorhinis in embryonated eggs did not give distinct end points (166). Embryos which survived longer than 4 to 6 days after inoculation developed characteristic lesions of myocarditis, pericarditis and occasional peritonitis (164, 185).

Infected chicken embryo tissues and associated membranes and fluids were found to contain high concentrations of the organism (36, 166). Embryonated hens' eggs were found to be slightly inferior to cell cultures or some types of cell free media for propagation of Mycoplasma hyorhinis (72, 161, 184) but not to commercial mycoplasma media (41).

Mycoplasma hyorhinis grew readily in media with a fresh beef heart infusion base (70, 82, 97, 99, 145, 163). Commercial desiccated type media were also used to propagate the organism (41, 43, 46, 59, 94) but they were found to be inferior to cell cultures (41, 99) or fresh beef heart infusion supplemented with turkey (70, 146) or swine (99) serum. Other media such as thioglycollate broth-infusion agar (18), Edward's medium (13), Hayflick's medium (14), horse flesh bouillon with neopeptone (43) and one consisting of Hanks' balanced salt solution with 20 per cent yeast extract and 10 per cent commercial mycoplasma medium (62) were used to grow the organism. A concentration of 20 per cent avian serum (145, 163), horse serum (18, 62, 72), rabbit serum (13) and swine serum (99) was incorporated in all these basal media. One investigator however, used 10 per cent serum in media and found that at this concentration swine serum was superior to turkey, chicken and

rabbit sera (97). Serum was found to be essential for the propagation of Mycoplasma hyorhinis (13, 97, 163, 181), but it was substituted successfully in one instance by 30 per cent human ascitic fluid (82).

Supplementation of basal media with yeast (43, 62, 72, 97), hemoglobin (163), peptone (70) or neopeptone (43) was reported to enhance growth but gastric mucin (97, 145), ribonucleic acid (97) and deoxyribonucleic acid (97) did not enhance the growth of the organism.

In fluid medium, the first indication of growth by Mycoplasma hyorhinis was the development of faint turbidity in the upper half of the medium (82, 145, 163). A slight sediment (163) and uniform turbidity (82, 145, 163) was observed in older cultures, but pellicle formation was not seen (163).

Typical mycoplasma colonies developed on solid media prepared by the incorporation of agar in fluid media (13, 18, 41, 46, 72, 82, 94, 97, 166). Nobel agar was found to be superior to other types of agar for this purpose (145). The colonies were small, glistening, had regular margins, did not develop at a uniform rate, had little tendency to coalesce and the central elevation was small and indefinite or missing in some cases (163). The colonies became embedded making them difficult to transfer (41). Older colonies developed a granular surface (41). Colonial growth in fluid medium was observed on plastic surfaces in the presence of high dilutions of specific rabbit antiserum (60).

Mycoplasma hyorhinis exhibited optimum growth under aerobic conditions (2, 133, 145, 162), but some strains preferred an anaerobic atmosphere especially for initial propagation (13, 18).

The so called "film and spots" reaction did not develop on solid media on which the organism was grown (2, 14, 166). Satelliting of nurse colonies was observed on solid media of the commercial desiccated type (41, 60, 94) or media containing a low concentration of serum (97).

Mycoplasma hyorhinis was isolated and propagated in various cell cultures (table 1). A cytopathic effect developed in the cell cultures beginning with cytoplasmic granulation (3, 13, 57, 171, 184), followed by shrinking (13, 57, 184), fragmentation (171) and syncytial development or extrusion of the cytoplasm (171). Eventually the cell sheet became a network of damaged and disintegrating cells (171) or clumps and clusters of cells developed which were inclined to become detached (3, 13). The nucleus became involved in advanced stages of infection and prominent refractile vacuoles were observed in both the cells (3) and the medium (84). In stationary tube cell cultures the cytopathic effect started at the base of the cell sheet and spread rapidly to form a characteristic "V" shape (57, 84, 148, 171). The cytopathic effect developed more rapidly and was more severe in certain cell cultures (3, 13, 57). The organism had little effect on primary monkey kidney cells (13). Cytopathic effects did not develop in cell cultures inoculated with certain isolates of the organism (43, 100) or when small inocula of

cytopathic strains were used (3). In primary swine testicle cell cultures the cytopathic effects of Mycoplasma hyorhinis and Mycoplasma hyopneumoniae were similar (99). Arginine supplementation of cell medium resulted in a deminished cytopathic effect by the organism (3).

Morphology and staining properties

Mycoplasma hyorhinis was passed through Selas 02 filters (17, 164) and 300 nm cellulose acetate filters (153, 168). Contradictory reports exist on the ability of the organism to pass through 220 nm cellulose acetate filters (161, 168). Selas 03 (164), wood pulp (164), asbestos fiber (164), and Berkeveld-N (116) filters reduced the titer and Seitz EK (36), Seitz ST (36) and Mandler filters of 7 pounds bubbling pressure (164) retained the organism.

The organism was stained with Giemsa (13, 43, 70, 84, 99, 145, 164, 185), Macchiavello stain (164), Stevenel blue (145), methylene blue (18), May-Greenwald stain (84), acridine orange (88), Dienes' stain (43) and orcein (43). The organism was found to be gram negative (164, 185) and non acid fast (164).

Light microscopic examination of stained preparations of Mycoplasma hyorhinis indicated that it was very pleomorphic (70, 84, 88, 166) consisting of round to ovoid shapes 200 to 600 nm in diameter (36, 41, 82, 84, 88, 99, 164, 185), longer rods up to 1 μm in length (13, 97, 166) and, in cell culture, large intercellular aggregates 5 to 6 μm in diameter (84). Electron microscopy revealed that these aggregates consisted of up to 50

Table 1. Cell cultures in which *Mycoplasma hyorhinis* was found to grow

Cell culture	References
Primary swine lung	159, 174, 184
Secondary swine lung	159
Primary swine kidney	100, 159, 174, 184
Secondary swine kidney	159
Primary swine testicle	99, 174
Secondary swine nasal mucosa	159
Primary bovine skin	184
Primary bovine kidney	43, 174
Primary bovine testicle	174
Primary chicken embryo	57, 174
Secondary chicken embryo	3
Chicken embryo fibroblasts	3
Primary rabbit kidney	13
Rabbit kidney cell line	3
Primary monkey kidney	3, 13, 57, 88
Primary guinea pig embryonic kidney	3
Mouse cell line L-MCN	3
Hampster sarcoma cell lines: HT55, 2HT	3
Hampster embryo fibroblasts	154
HeLa cell line	3, 13
HEp-2 cell line	13, 148
Primary human embryonic kidney	3, 57, 154

Table 1 (Continued).

Cell culture	References
Primary human amnion	3
Various human diploid cell lines	3, 57, 76, 88

individual particles (84).

Switzer (166) failed to demonstrate filamentous forms in broth cultures, but others reported transitory filamentous shapes (54), filaments budding off ovoid bodies (88), dense nucleoids joined up by fine filaments (84) and filaments with darker spheres resembling streptococci (18).

Biochemical and metabolic activity

Mycoplasma hyorhinis rapidly reduced triphenyl tetrazolium chloride (2, 145, 166), tellurite (2) and methylene blue (2, 145) in fluid medium but not methyl red (119). The rate of tetrazolium reduction varied with different isolates (70).

Early work on the organism indicated that several carbohydrates, including glucose, were not metabolized (13, 82, 166), but in more recent work the organism was shown to utilize glucose (3, 57, 96, 168, 175, 181) by an oxidative pathway (2). In beef heart infusion medium several passages of the organism in the presence of glucose were required before utilization of the sugar could be detected (168). Indications of glucose fer-

mentation by the organism were not seen in Edward's semi-solid medium but it could be demonstrated in Hayflick's medium (96). In other work prior conditioning of growth medium by Escherichia coli was required to demonstrate glucose and maltose fermentation (126). The organism metabolized mannose (181) but not arginine (2, 133, 175). It was found to possess the arginine dihydrolase pathway but not arginine deiminase activity (8). The organism was found to possess phosphatase activity (2). Acidification resulted from growth of the organism in cell cultures (3, 13, 43, 57, 84, 154, 159) and fluid medium (119, 129).

Mycoplasma hyorhinis produced beta hemolysis of guinea pig erythrocytes (3, 14, 57) and alpha hemolysis of horse (13) and sheep (2) erythrocytes. Consistent hemagglutination was not observed with bovine (175), guinea pig (175), swine (142), sheep (142), fowl (175, 184), human (175), monkey (175), mouse (142, 175), rabbit (175), rat (142, 175) or turkey (175) erythrocytes, but in one report it was claimed that positive results were obtained with swine, rabbit, sheep and fowl erythrocytes (46). Hemadsorption by the organism was not demonstrated with bovine, guinea pig, fowl, human, monkey, mouse, rabbit, rat and turkey erythrocytes (175). Carotenoid pigments were not produced by Mycoplasma hyorhinis (181). The organism did not hydrolyze gelatin nor did it digest casein or serum (2).

Antigenic properties and serology

Techniques that were used for the identification and serological study of Mycoplasma hyorhinis include neutralization

(36, 88, 148), agglutination (36, 126, 152), agar diffusion precipitation (41, 93, 148), indirect hemagglutination (119, 145), growth inhibition (29, 57, 61, 80, 93, 126, 152), complement fixation (11, 57, 93, 96, 132) and immunofluorescence (10, 14, 40, 179).

The neutralization procedure in cell cultures (148) and the growth inhibition technique (80, 93) were found to be strain specific. However, growth of some isolates of the organism was not inhibited by homologous rabbit antiserum (41, 80). Mycoplasma hyorhinis was shown to share some antigens with Mycoplasma canis, Mycoplasma fermentans, Mycoplasma neurolyticum and Mycoplasma pulmonis by agar diffusion precipitation and complement fixation but not by growth inhibition (93).

Several investigators were unable to detect an immune response to Mycoplasma hyorhinis in swine (145, 162, 184). Recently, the metabolic inhibition test was employed to detect antibodies in sera of experimentally (4, 64) and naturally (59) infected swine. Similar results were obtained using a modified direct complement fixation test in which guinea pig complement was supplemented with fresh calf serum to demonstrate antibodies in sera from experimentally infected swine (11).

Sensitivity to antibacterial and physical agents

Mycoplasma hyorhinis was resistant to penicillin (13, 36, 57, 97, 145, 164), thallium acetate (13, 145, 166), erythromycin (36), kanamycin (148) and bacitracin (164) in vitro. Moderate susceptibility to streptomycin was demonstrated (13, 36, 97,

164). The organism was sensitive, in vitro, to chlortetracycline (36, 57, 88, 97, 153, 184), oxytetracycline (36, 97, 164, 184), tetracycline (13), tylosin (43, 161), chloromycetin (97), lincomycin (168), ethyl alcohol (166), ether (88) and optochin (2).

Antibiotic treatment of infected swine was found to be of little value (94, 127, 161) but the prophylactic use of tylosin gave promising results (161). Limited success was reported with therapeutic use of high levels of sulfonamides (17, 162), tetracycline (82) and oxytetracycline (82, 124).

The organism was found to survive 56°C for at least 30 minutes (163), 37°C for 3 days (153), 22°C for 1 week (153), 10°C for 3 days (153), 5°C for 6 to 7 weeks (153), 4°C for 2 to 3 weeks (164) and -40°C for at least 5 years (161). It survived lyophilization for over 5 years when stored at 4°C (161). One investigator found that a tissue culture strain of the organism was inactivated by 56°C within 3 minutes (88).

Mycoplasma hyorhinis was stable between pH 7.4 and 8.0 (36) but was killed at pH 3 (88). Rapid changes in ambient osmotic pressure (36) and three successive cycles of rapid freezing and thawing (13) did not have a marked detrimental effect on the organism.

Pathogenicity and epizootiology

A polyserositis-arthritis syndrome in young pigs was attributed to infection with Mycoplasma hyorhinis by several investigators (74, 94, 97, 116, 164, 185). Experimentally, pigs were

susceptible up to 6 to 8 weeks of age (97, 164, 185), but under natural conditions the disease was observed in pigs up to 10 weeks of age (164) and occasionally in adult swine (82, 167). Mycoplasma hyorhinis was observed to have a greater affinity for serosal membranes of infected pigs than for synovial membranes (162), but strains varied with respect to their arthrotropism (115, 162). The disease was reproduced in susceptible pigs with infective material by intraperitoneal (4, 74, 95, 97, 116, 127, 165), intrathoracic (116, 127), intravenous (162) and subcutaneous inoculation (116). Intranasal (74, 165) and intracerebral (116) inoculations were generally unsuccessful but one investigator claimed successful results with intranasal inoculation (58). Mycoplasma hyorhinis generally retained its virulence for pigs after many passages in artificial media (169) but the arthrotropic potential of some strains became attenuated after several passages in chick embryos (115).

White mice (116, 164), rabbits (116, 164), guinea pigs (116, 164), chickens (164), turkey poults (164), a calf (163) and lambs (166) were not susceptible to the organism. A mycoplasma responsible for septicemia and arthritis in goats produced polyserositis, arthritis and leptomeningitis in experimental pigs, but the relationship of this organism to Mycoplasma hyorhinis was not established (37).

Mycoplasma hyorhinis was frequently isolated from the nasal cavity of swine (17, 43, 70, 71, 94, 147, 150, 164), and from lungs of swine with enzootic pneumonia (19, 46, 59, 73, 100,

150, 153, 163, 184), but less frequently from normal lungs (19, 59, 73, 100). It was not isolated from the nasal cavities of 80 humans with a high incidence of swine contact (172). Although the organism was frequently isolated from diseased lungs and nasal cavities of swine, most investigators found that it did not produce atrophic rhinitis (20, 74, 165) or pneumonia (20, 74, 150, 153, 162) under experimental conditions. Several investigators produced pneumonia in pigs by inoculation of pneumonic lung tissue or surviving lung cells yielding Mycoplasma hyorhinis into the respiratory tract but failed to do so after laboratory propagation of the organism (20, 95, 143). One investigator however, reported experimental production of transient rhinitis and mild but definite pneumonia in pigs by intranasal inoculation of the organism (62).

The organism was isolated from visceral lesions during the acute phase (4, 139) and from affected joints during the acute and chronic phases (4, 5, 140) in pigs with experimental infection. In one case it was isolated from the cerebrospinal fluid (139).

Mycoplasma hyorhinis superinfection did not aggravate Hemophilus influenza suis serositis (97), erysipelas arthritis (116) or Pasteurella multocida pneumonia (138) in pigs. Experimental evidence indicated that polyserositis due to Mycoplasma hyorhinis had little effect on the fertility of boars (167).

Outbreaks of Mycoplasma hyorhinis polyserositis in swine were associated with various predisposing stress conditions (17,

97, 115, 161). The most severe cases were often observed to have concurrent anemia (166). It was estimated that the organism gained entrance into susceptible pigs from damaged turbinates or diseased lungs (19, 162). Growth was stunted in pigs with mycoplasmal polyserositis (4, 167) and 5 to 10 per cent of market weight swine exhibited residual lesions of the disease.

Pathology

Polyserositis in swine due to Mycoplasma hyorhinis was characterized in the early stages by fibrinous (82, 164), serofibrinous (94, 185) or fibrino-purulent (139) exudate. Older lesions consisted of pasty fibrinous deposits causing varying degrees of attachment between the involved organs (94), which eventually developed into fibrous adhesions (94, 161). Histologically, this exudate consisted of fibrin and neutrophils with a few macrophages and lymphocytes (139). Serosal membrane cells were swollen (139) and the membrane became infiltrated with plasma cells, macrophages and occasional neutrophils. These cells were replaced with lymphocytes by 30 days post inoculation (139). The inflammatory changes on the serosa of various organs extended a short distance into the organ parenchyma (139).

Arthritic joints were sometimes noticeably swollen due to soft fluctuating distention of the joint capsule (140). Early lesions of the synovial membrane included hyperemia (140), edema (94, 116, 140), fibrin deposits on the surface (140) and hemorrhage (116). An increased amount (116, 140, 164, 185) of

turbid (116, 140, 185), mucinous (116, 140), occasionally serous (115), blood tinged (116) synovial fluid was observed. Periarticular edema occasionally developed (115). Early histological lesions were described as hyperemia of the synovial membranes, enlargement of the synovial membrane cells and infiltration of the membranes by plasma cells, mononuclear macrophages, a few lymphocytes and occasional neutrophils (140). Electron microscopy revealed fibrin deposits on the surface and in the superficial intercellular area of the synovial lining. Neutrophils infiltrated between lining cells. Hyperplasia of type A synovial lining cells and hypertrophy of endothelial cells were observed and the type A cells contained phagocytosed neutrophilic granules and various types of membrane bound vesicles (45). The organism could not be detected in affected synovial membranes by electron microscopy (45) but electron microscopic examination of infected cell cultures revealed organisms attached and fused to the plasmalemma, single or in groups within vacuoles in the cytoplasm and free in the cytoplasm of cells (84). Localized cellular damage was observed in the vicinity of free mycoplasma particles but not around mycoplasma-containing vacuoles (84). The neutrophil was the principle cell encountered in synovial fluid of infected joints (45, 140).

As the interval after inoculation increased, plasma cells (4, 45) and lymphoid type cells (4, 45, 115, 140) increased, especially around blood vessels, but depletion of these cells

started about 56 days post inoculation (140). Marked hypertrophy of synovial villi (4, 115, 140) and hyperplasia of synovial cells (140) were observed in chronically affected joints. Pannus formation in articular cartilages developed occasionally (140). Bone changes, starting about the 10th day post inoculation, were characterized by disorganization of the orderly maturation of cartilage cells in the vesicular zone of the epiphyseal plate resulting in osteoporosis and osteolysis (140). Fibrosis of the synovial membrane was not generally observed (45, 140), although one worker reported finding this lesion (4) and periarticular fibrous proliferation of chronically affected joints was described by another investigator (115).

A slight lymphocytic reaction developed in the nasal mucosa of swine after intranasal instillation of the organism (164) and a lymphocytic leptomeningitis was observed in one experimental pig (139).

Association with cell cultures and tumors

Several investigators used cell cultures for the isolation and propagation of Mycoplasma hyorhinis strains of swine origin (43, 100, 142, 159, 174). Strains of mycoplasma were isolated from uninoculated cell cultures (13, 57, 88), from cell cultures prepared from or inoculated with material from various solid tumors (2, 148) and from a bladder papilloma (77). These tumors and cell cultures are listed in table 2. These myco-

Table 2. Isolation of Mycoplasma hyorhinis from cell cultures and tumors

Tumor or cell culture	Reference
HEp-2 cell line	13
Human diploid cell lines	57, 88
Primary African green monkey kidney	57
Primary chicken embryo	57
Retropharyngeal thymoma	3
Hemangioma	3
Thymoma	148
Papilloma (bladder)	77

plasmas were found to be related to Mycoplasma hyorhinis by serological techniques (132, 179), nucleic acid homology (135, 156) and analysis of the guanine-cytosine content (114).

In recent years Mycoplasma hyorhinis was the most common mycoplasmal cell culture contaminant, possibly due to contaminated batches of trypsin (76). The organism was not always easily recovered from cell cultures, apparently because of its adaptation to intracellular growth (14).

Stanbridge, quoted by Hayflick (76), observed aberrations of chromosomes such as chromatid breaks, achromatic gaps, dicentrics, ring chromosomes and pulverizations in chronically infected cell cultures.

The organism did not induce interferon production in various cell cultures (3), but prevented interferon production in cell cultures by two interferon producing agents (154).

Mycoplasmas were detected both intracellularly and extracellularly in cell cultures (13, 84, 88, 159), although some strains exhibited intracellular growth only (14, 171, 185).

Mycoplasma Granularum

History

A serologically homogenous group of mycoplasmas, isolated from swine joints, which differed physiologically, morphologically and serologically from Mycoplasma hyorhinis, was described by Ross and Switzer in 1963 (145).

Switzer selected the name of Mycoplasma granularum for these strains because of their distinctive growth characteristics in fluid medium (161). Subsequently several reviews were written which included information on this organism (146, 167, 168, 169, 170).

Recent investigation has revealed considerable heterogeneity among strains designated Mycoplasma granularum with regard to their physiological, morphological and antigenic properties (144). Undoubtedly, a review of the literature at this point would expose many contradictory observations and a review at a later date when more clarity has been obtained as to the identity of these strains would be of great value.

Laboratory propagation and growth characteristics

Mycoplasma granularum was found to grow slowly in a fresh beef heart infusion broth with 20 per cent turkey serum (145). A commercial desiccated type medium was found to be superior to a fresh beef heart extract (70, 143). This type of medium was used by several investigators to propagate the organism (41, 43, 175, 179). Other media that were used included Hayflick's medium (179), Edward's medium (180), nutrient bouillon (56), horse meat bouillon with neopeptone (43) and a medium consisting of Hank's balanced salt solution with 20 per cent fresh yeast extract and 10 per cent Bacto beef heart infusion (62).

Dinter et al. (41) reported that serum was essential for growth of Mycoplasma granularum. Turkey serum (145) or horse serum (43, 56, 58, 175, 179) at a concentration of 20 per cent and turkey serum at a concentration of 15 per cent (70, 143) was incorporated in the media employed for propagation. However, Mycoplasma granularum (strain 39) grew in media from which the serum was omitted (181) or replaced by PPLO serum fraction (179).

Gastric mucin (145) and yeast supplementation (43, 62, 145, 175, 179) of medium enhanced growth of Mycoplasma granularum. Cholesterol (180), Tween 80 (180) or agitation (145) did not improve growth of this organism.

Growth in fluid medium was characterized by uniform turbidity (145) with a slight granular texture (161), waxy pellicle (161) and a granular deposit (145). Rapid growth and little

granularity was noted with some strains (179). In semi-solid medium the organism grew at all depths along a stab (145). Colonization of Mycoplasma granularum on plastic surfaces in liquid medium was observed in the presence of high dilutions of specific rabbit antiserum (60).

Colonies typical of mycoplasma developed on solid medium (41, 43, 145, 161, 179). The colonies were very similar to those of Mycoplasma hyorhinis (145) except for a larger central area (161). A recent report indicated that colonies of Mycoplasma granularum were typically granular and could be differentiated from the smooth colonies of Mycoplasma hyorhinis (43). Growth of this organism on solid medium was associated with the development of a film imparting a metallic sheen (170). However, strain 39 did not exhibit "film and spots" formation (2). Satelliting with staphylococcus colonies was not observed (41).

Mycoplasma granularum did not grow in embryonated hens' eggs (41, 168) nor did it have a distinct cytopathic effect on primary swine (142, 169) or calf (41) kidney cells. However, one investigator reported development of a cytopathic effect in calf kidney cells (43) although strains that were isolated in cell free medium had to be adapted to grow in these cell cultures (44).

Biochemical and metabolic activities

Mycoplasma granularum did not reduce triphenyl tetrazolium chloride in fluid medium (43, 70) and reduction of methylene blue was slow (145). However, Mycoplasma granularum strain 39

was recently shown to reduce methylene blue and tetrazolium anaerobically and tellurite aerobically (2). Occasional decoloration of phenol red was observed (170).

Mycoplasma granularum fermented glucose (2, 170, 175, 179) but not mannose (181). One investigator found that the organism had to be adapted to glucose containing medium before the sugar was noticably fermented (170). Others showed that the organism acidified fluid media (119, 129) and cell cultures (43, 168). Arginine was not metabolized by strain 39 (2, 175) nor was phosphatase activity detected in this strain (2). The prototype strain 39 and three other strains produced carotenoid pigments (181) and did not incorporate cholesterol during growth (180).

Strain 39 did not hydrolyze gelatin nor did it digest casein or serum (2). Two contradictory reports on the ability of this organism to hemolyze erythrocytes were recorded (43, 170). Mycoplasma granularum did not hemadsorb or hemagglutinate bovine, guinea pig, human, fowl, monkey, mouse, rabbit, rat or turkey erythrocytes (108).

Morphology and staining

Mycoplasma granularum has been passed through Selas 02 (168), 300 nm (168) and 220 nm cellulose acetate filters (172), but not through 100 nm cellulose acetate filters (172).

The organism was stained by Giemsa (43, 145), Stevenel blue (145), orcein (43) and Dienes' stains (43).

Individual organisms stained with giemsa appeared predominantly as coccoid rods 1.3 to 1.6 μm in length (161). Ring

forms were not observed (170). In Giemsa stained smears of culture sediment blue bodies 5 to 15 μm in size were seen (145) which apparently formed around clumps of organisms (161). Low passage isolates of Mycoplasma granularum were noted to be more uniform in morphological features and staining properties than low passage isolates of Mycoplasma hyorhinis (70).

Antigenic properties and serology

Serological techniques used to identify Mycoplasma granularum and to differentiate it from other species of mycoplasma included indirect hemagglutination (119, 145), gel diffusion precipitation (41, 93), agglutination (152), indirect immunofluorescence (179), direct immunofluorescence (181), growth inhibition (41, 61, 93, 152, 181), metabolic inhibition (59, 175) and complement fixation (93, 132, 180).

Mycoplasma granularum has been shown to be related to Mycoplasma laidlawii by indirect immunofluorescence (181), complement fixation (93, 180), agar diffusion precipitation (93), growth inhibition (181) and electrophoresis of cell proteins in polyacrylamide (181). These two species were shown to be antigenically distinct by direct immunofluorescence (181), growth inhibition (93, 152, 180) and agglutination (152).

Serological detection of antibodies to Mycoplasma granularum in sera from infected swine has not been reported (59, 145, 175). Warm type agglutinins against human O erythrocytes and anti rabbit gamma-globulin were demonstrated in sera from infected swine (1).

Sensitivity to antibacterial and physical agents

Mycoplasma granularum was found to be sensitive to tylosin (56, 170) and lincomycin (146) in vitro but not to penicillin and thallium acetate (145). In vitro resistance to tetracycline, kanamycin, erythromycin, chloromycetin, polymixin, stovarsol and iodine has also been demonstrated (56). Digitonin did not effect the growth of strain 39 except at levels above 500 µg per ml (180).

Infected swine were amenable to treatment with tylosin tartrate (161) and lincomycin (169). Incidence of the disease in a herd was reduced with tylosin administration but nasal carriers were not eliminated (161).

The organism survived lyophilization and remained viable for several years in the frozen state at -25°C (168).

Pathogenicity and epizootiology

Mycoplasma granularum produced arthritis in adult pigs but did not produce disease in young pigs after intravenous (161) or intraperitoneal inoculation (170, 179). Pigs weighing 65 to 130 pounds were the most susceptible but those from 130 to 220 pounds also developed the disease (146).

Mycoplasma granularum was estimated to be the most common organism isolated from acute non-febrile non-polyserositis-associated arthritis in swine (170). The fertility of affected boars was reported to be impaired after infection with the organism (169). Experimental production of the disease was often unsuccessful or erratic (127, 161) but the use of an improved

medium to propagate the organism markedly enhanced its virulence for swine (143). Intratracheal inoculation did not elicit pneumonia in swine (161).

Mycoplasma granularum was isolated from arthritic joints (145), regional lymph nodes (143), nasal mucosa (43, 145), lungs (59, 145) and occasionally from the tonsils (142) of swine. In experimentally infected swine the organism was isolated from joints and lymph nodes only during the acute stages of the disease (143). No correlation was established between the occurrence of pneumonia or atrophic rhinitis and the development of arthritis due to the organism (161).

The disease was seen to occur endemically in herds with 5 to 20 per cent of the animals affected, but occasionally a morbidity of up to 50 per cent was encountered (161). Strong circumstantial evidence indicated that predisposing stress (127, 146) and genetic background of the pigs (167) played an important role in occurrence of the disease. Most pigs recovered within 2 weeks but 10 to 20 per cent of the cases were found to persist (167).

Pathology

The macroscopic and histologic appearance of the arthritis associated with this disease was described by Ross and Switzer (146). They noted an acute periarticular and peritendinous edema and the frequent distention of joint capsules. Excessive, turbid, serofibrinous or serosanguineous synovial fluid with occasional tags of fibrin was often present in affected joints

in the acute stage of the disease. The synovial membranes were hypertrophic, hyperemic, edematous, and discolored reddish brown. Histologically, hyperplasia of synovial lining cells was seen and perivascular or diffuse accumulations of lymphocytes, plasma cells and some neutrophils were observed in the synovial membranes.

In chronic cases serosanguineous synovial fluid was often present in joints and the synovial membranes were markedly hypertrophic with a yellow to tan color (146). Pannus formation (146), articular cartilage necrosis (146), flattening of the articular surface (168) and mild to moderate periarticular fibrosis were sometimes seen (168).

Genetics and association with cell cultures

A mycoplasma isolated from a murine leukemia cell line (56) was identified serologically as Mycoplasma granularum (179).

Nucleic acid homology was used to differentiate Mycoplasma granularum from other species of mycoplasmas (156). The nucleic acid buoyant density of this organism was established at 1.6850 g per cc (156) and 1.6915 g per cc (92). The guanine-cytosine content was found to be 30.4 per cent (114) and 32.1 per cent (92).

Immunofluorescence with Mycoplasmas

History

Since a vast amount of literature has appeared on immunofluorescence since its inception by Coons in 1942 (33) this review will be limited to application of immunofluorescence to the study of mycoplasmas.

The first use of the fluorescent antibody technique in the study of a mycoplasma was made by Liu and Eaton when they demonstrated the Eaton agent in the bronchial epithelium of infected chicken embryos (103). Since this initial work immunofluorescence has been used for differentiating mycoplasmas in fluid (10, 15, 112, 179) or solid (21, 24, 35, 40, 157, 181) media, to study their growth in cell cultures (7, 14, 16, 24, 27, 28, 48, 107), to follow the pathogenesis of mycoplasmal diseases (9, 10, 15, 35, 91, 123, 125, 128, 141, 151, 182) and for detection of specific mycoplasmal antibody (22, 29, 30, 31, 65, 103, 105, 106, 176). Two reviews have been published which deal with mycoplasmas and immunofluorescence (47, 131).

Mycoplasmas and cell cultures

The direct fluorescent antibody test was used on several occasions to detect mycoplasma contaminants of cell cultures (7, 16, 107).

Human mycoplasmas

The indirect fluorescent antibody technique was a significant contribution to the study of *Mycoplasma pneumoniae*. The

technique was developed by Liu who was able to recognize the organism in frozen sections of infected chick embryo lungs (101, 103, 104). His technique was used by other workers with little modification in epidemiology studies (22, 23, 30, 31), to identify the organism (40, 58), to detect specific antibodies in human convalescent serum (29, 65, 103, 105, 106, 176) and rabbit hyperimmune serum (176) and to monitor the effect of antibacterial agents against the organism (49, 110).

The indirect procedure was used to detect (27, 48) and to quantitate (28) Mycoplasma pneumoniae in cell cultures and to identify preparations of the organism grown on solid medium (21, 157) or in broth cultures (179). It was used to demonstrate the organism in the bronchial epithelium of experimentally infected hamsters (39) and sputum or throat washings of infected humans (83).

Both the indirect and the direct immunofluorescence tests were satisfactory for differentiation of Mycoplasma hominis I from Mycoplasma hominis II grown on solid medium or in cell cultures (24). The indirect method was employed to differentiate several other species of mycoplasmas of human origin grown in fluid medium (179) and the direct method was used to identify human mycoplasmas grown on solid medium (40).

Swine mycoplasmas

The indirect fluorescent antibody technique was used to identify Mycoplasma hyorhinis in broth cultures (179) and cell

cultures (14), Mycoplasma granularum in broth cultures (179) and Mycoplasma hyoarthrinosa in the tissues of experimentally infected swine (141).

The direct immunofluorescence technique was employed to identify colonies of Mycoplasma hyorhinis (40) and Mycoplasma granularum (181) on solid media, to recognize Mycoplasma hyorhinis in cell cultures (88) and to differentiate Mycoplasma hyopneumoniae from Mycoplasma hyorhinis in broth cultures (10). Mycoplasma hyopneumoniae was detected in lungs of swine with enzootic pneumonia by direct immunofluorescence (10).

Mycoplasma granularum was shown to be related to Mycoplasma laidlawii by means of the indirect fluorescent antibody technique but this relationship was not bourne out by the direct test (181).

Avian mycoplasmas

Direct immunofluorescence was used to identify Mycoplasma gallisepticum in solid (35, 128) and liquid cultures (15), in the tissues of experimentally infected turkeys (123, 128), chickens (15, 123, 151) and cynomolgus monkeys (158) and in tissues of naturally infected turkeys (15, 35, 123) and chickens (9, 15, 35, 123). This technique was also used to distinguish colonies of Mycoplasma synoviae and Mycoplasma gallisepticum (35).

Indirect immunofluorescence was employed to recognize Mycoplasma gallisepticum in tissues of naturally infected chickens and turkeys (123) but was unsatisfactory for identification

of colonies of the organism (35).

Murine mycoplasmas

Indirect immunofluorescence was used for the localization of Mycoplasma pulmonis in the bronchial epithelium of infected mice (125, 182) and to differentiate Mycoplasma pulmonis, Mycoplasma neurolyticum, Mycoplasma arthritidis and Mycoplasma histotropicus (177). An antigenic relationship between Mycoplasma arthritidis and Mycoplasma hominis type II was demonstrated by indirect immunofluorescence (177).

Bovine mycoplasmas

Direct immunofluorescence was used to identify Mycoplasma mycoides grown in fluid medium (112), to demonstrate Mycoplasma agalactiae var. bovis in smears made from infected milk (90) and to localize the latter organism in infected mammary tissue (91).

Sensitivity

The sensitivity of the indirect fluorescent antibody technique was enhanced by supplementation of human convalescent sera with fresh guinea pig or human serum (48, 64, 102), but this phenomenon could not be demonstrated with rabbit hyperimmune sera (102).

Immunofluorescence was generally found to be as sensitive (7) or more sensitive (24, 40, 158) than cultural procedures for detection of mycoplasmas in cell cultures or clinical materials except that one investigator reported slightly better

results with isolation techniques (128). The direct fluorescent antibody test was found to be less sensitive than the indirect test for detection of Mycoplasma pneumoniae in the lungs of infected chicken embryos (101).

Reactive antigens

Mycoplasma antigens reactive in immunofluorescence tests were located mainly in the cell membrane (40) and retained their activity after treatment with 1 per cent acetic acid, 1 per cent hot formalin and ethanol ether (24). Exposure to 10 per cent formalin (15) and excessive heat (24, 35) resulted in quenching of fluorescence.

The antigens retained their specificity after ultrasonic treatment and they were found mainly in the insoluble fraction (24). Mycoplasmal antigens liberated by alkaline hydrolysis and detectable by indirect immunofluorescence could be adsorbed onto tanned human erythrocytes (178).

Technique modifications

Whole serum (35, 123), euglobulin (112) and ammonium sulfate precipitated globulins (14, 24, 40, 87, 91, 128, 158) were used for conjugation with a fluorochrome. Hyperimmune serum produced in rabbits was utilized by most investigators (7, 15, 16, 24, 35, 88, 91, 123, 128, 151, 158), but bovine (112), turkey (35), chicken (35), horse (40), mule (40) and goat (40) sera were also used.

In most cases, the fluorochrome fluorescein isothiocyanate

(FITC) was conjugated to antibody protein with FITC to protein ratios of 1:20 (123, 158), 1:30 (15), 1:40 (35, 128), 1:70 (40) and 1:200 (87). Rhodamine sulphochloride was also used (151). Gel filtration with Sephadex G-25 (40, 87, 91, 128, 158, 160) and Sephadex G-75 (112) was employed to remove unreacted FITC from preparations of conjugated proteins.

Many modifications were adopted to improve the specificity of the technique. These modifications included absorption of FITC conjugated protein preparations with various tissue powders (7, 16, 35, 48, 64, 87, 101, 157, 158), activated charcoal (64) and washed yeast cells (157). Other modifications were dilution of conjugates (40, 48, 87), DEAE cellulose treatment of conjugates (35, 91), counterstaining with azo dyes (15, 123, 128) or with lissamine-rhodamine (7, 91, 107) and filtration of conjugates through cellulose acetate filters (87).

Reduction of the reaction time between conjugates and antigens (48, 87) and agitation of preparations during washing procedures (87, 157) were also employed to minimize non-specific fluorescence. Mycoplasmas were grown in media containing serum from the animal species used for immunization to curtail non-specific staining (16, 40).

Bluish autofluorescence was encountered by some investigators with preparations of mycoplasmas grown on solid media (24, 40).

Fixation of mycoplasma specimens on glass slides was achieved by treatment with methanol (15, 24, 112, 179), ethanol

(15, 24), hot water (24, 128, 157), buffered formalin (7, 107) and acetone (16, 35, 48, 64, 101, 157) or by air drying with gentle heat (35, 123, 128, 177, 182). Other investigators encountered poor fixation of culture material on slides with acetone (15, 24) and ethanol (15). Prolonged fixation with acetone (157) and hot water fixation of colony impressions (35) resulted in relatively poor fluorescence. The growth of mycoplasmas on cellulose acetate membrane discs was a unique technique which was suitable for use in the fluorescent antibody test (107).

MATERIALS AND METHODS

Source of Mycoplasma Species

The source and origin of the mycoplasma strains used in this study are listed in table 3.

Propagation of the Organisms and Antigen Production

The group of mycoplasmas typified by Mycoplasma granularum strain S16 were grown in complete PPLO medium consisting of 85 per cent Bacto PPLO broth[1], 0.5 per cent Bacto mucin[1] and 15 per cent heated turkey serum (70). Mycoplasma meleagridis, Mycoplasma synoviae and Mycoplasma gallisepticum were propagated in FM4 medium (55) with 15 per cent rabbit serum. Mycoplasma hyopneumoniae was grown in a medium described by Maré and Switzer (109). All other mycoplasma strains as well as Mycoplasma gallisepticum and Escherichia coli were propagated in a medium consisting of fresh beef heart infusion, 1 per cent Bacto peptone[1], 0.2 per cent Bacto hemoglobin[1] and 15 per cent heated turkey serum. This medium is a modification of one described by Ross and Switzer (145).

Penicillin (1000 units per ml) and thallium acetate (1:4000) were included in media used for propagation of antigens for absorption and immunization and for isolation procedures. Mycoplasmas for infection of experimental swine and cell cultures were grown in broth media devoid of bacterial inhi-

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[1]Difco Laboratories, Detroit, Michigan.

Table 3. Source and origin of mycoplasma strains

Mycoplasma species	Strain	Origin	Immediate source
M. granularum sterol-requiring[a]	S16	Swine joint	R. F. Ross
	S149	" "	"
	S10	" "	"
	33R	Swine nose	"
	1918	Swine joint	"
	2130	" "	W. P. Switzer
	3480	Swine tonsil	R. F. Ross
	43L	Swine joint	"
	Gubser	Swine joint	W. P. Switzer
	Jenette	Swine lung	R. F. Ross
	Baldwin	Swine joint	W. P. Switzer
	74BRR	Swine nose	R. F. Ross
M. granularum non sterol-requiring (171)	BTS39	Swine nose	R. F. Ross
	31B-1	" "	J. G. Tully
	Friend	Tissue culture, murine leukemia	"
	SD7	Swine nose	"
M. hyorhinis	BTS7	Swine nose	R. F. Ross
	SK76	" "	"
	GDL	Tissue culture	R. H. Purcell
	FS3	" "	"
	T7	" "	"
	69N	Swine nose	R. F. Ross
	31BRL	" "	"
	36BRL	" "	"
	85R	" "	"
M. laidlawii A	14089	Sewerage	"
M. laidlawii B	14192	"	"
M. arginini	G230	Mouse brain	R. F. Ross
M. arthritidis	13988	" "	"

[a]R. F. Ross, Vet. Med. Res. Inst., I.S.U., Ames, Iowa. Sterol-requiring *M. granularum* strains. Personal communication. 1969.

Table 3 (Continued).

Mycoplasma species	Strain	Origin	Immediate source
M. hyopneumoniae	11	Swine lung	K. Lam
M. meleagridis	8M92	Turkey air sac	M. L. Frey
M. iners	867Y	Chicken pericardium	"
M. gallisepticum	801	Turkey air sac	"
M. synoviae	1853	Chicken joint	"
M. gallinarum	862Y	Chicken trachea	"

bitors.

Antigens for absorption and immunization were prepared by inoculating 250 ml of medium with 10 ml of a 24 hour broth culture of Mycoplasma granularum (S16), Escherichia coli or Mycoplasma laidlawii A (14089) or a 36 hour culture of Mycoplasma hyorhinis (SK76). These cultures were incubated for 48 hours at 37°C on a platform shaker[1], checked for bacterial contamination by microscopic examination of gram stained culture smears and harvested in a refrigerated angle head centrifuge at 22,000xG for 20 minutes. Each harvest was washed twice with sterile physiological saline, centrifuged as before and then resuspended in sufficient physiological saline so that a 1:20 dilution had

[1]Eberbach and Son Co., Inc., Ann Arbor, Michigan.

an optical density value of 0.2 on a Coleman Junior spectrophotometer[1] set at 420 nm. Antigens were stored in 1 ml amounts at -70°C.

Medium for isolation procedures and for propagation of antigens for immunofluorescence was prepared in 5 to 6 ml amounts in 16x25 mm screw cap glass culture tubes. Immunofluorescence antigens were prepared by centrifuging 5 ml amounts of 24 to 48 hour cultures in conical tubes in a refrigerated swing-out head centrifuge at 3,400xG for 20 minutes. The supernatant was poured off and the tubes containing the sediment were allowed to drain for two minutes. In some cases the antigens were washed twice with 0.25M NaCl prior to centrifugation. The sediment was resuspended in 0.3 ml of sterile physiological saline and two drops of the suspension were placed on glass slides 3 cm apart. The sediments were air dried, fixed with absolute methanol for 10 minutes, washed one minute in deionized water and allowed to dry at room temperature. The antigen was carefully ringed with a Marktex paint pen[2] and then either examined immediately or stored for up to 1 week at 4°C before testing.

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[1]Coleman Instruments, Inc., Maywood, Illinois.

[2]Mark-Tex Corp., Englewood, New Jersey.

Production of Hyperimmune Sera

Intravenous inoculation

Two white New Zealand rabbits (Nos 055, 056), 2 Broad Breasted Bronze turkeys (Nos VD775, VD4278), 3 White Leghorn roosters (Nos VD1651, VD1652, VD2425) and a 6-month-old pig (No 4084B) were used. The rabbits were procured from a local breeder and the other species were raised at the Veterinary Medical Research Institute. A pre-inoculation sample was taken and the animals were given a series of six intravenous inoculations of strain S16 antigen diluted 20 times (O.D. 0.2), over a 2 week period followed by a single booster inoculation 12 weeks later. Blood was collected 10 days after the 6th inoculation and 13 days after the booster injection. The dosages administered to these animals were as follows:

Rabbits: 0.25, 0.5, 1.0, 2.0, 4.0, 4.0 and 3.0 ml.

Roosters: 0.2, 0.5, 1.0, 2.0, 4.0, 4.0 and 3.0 ml.

Turkeys: 0.5, 1.0, 2.0, 4.0, 8.0, 8.0 and 8.0 ml.

Pig: 2.5, 5.0, 10.0, 20.0, 22.0, 32.0 and 30.0 ml.

The same procedure was followed for production of *Mycoplasma hyorhinis* (SK76) antiserum. The booster inoculation was given 5 weeks after the last injection of the series and sera were collected 9 days after the 6th inoculation and 8 days after the booster. The same dosages were used for 2 white New Zealand rabbits (Nos 074, 075). Two eight-week-old pigs (Nos 4721G and 4722G) received 1.0, 2.0, 4.0, 4.0, 4.0, 8.0, 8.0 and a booster of 10.0 ml.

Subcutaneous inoculation

Two white New Zealand rabbits were given a series of three subcutaneous inoculations with strains S16 and SK76 antigen, emulsified in an adjuvant, at 9 week intervals. The antigens for the first 2 inoculations were prepared by emulsifying undiluted antigens with equal volumes of Freund complete adjuvant[1] which was then inoculated in 0.2 ml amounts into each rear footpad and at 3 separate subcutaneous sites, so that a total of 1 ml was administered to each rabbit. For the third inoculation, a twofold dilution of antigen was made in physiological saline and this was emulsified in Freund complete adjuvant using equal volumes of each. Each rabbit received 2 ml of this emulsion, 0.25 ml in each rear footpad, 0.2 ml at 5 separate subcutaneous sites and 0.5 ml intramuscularly. A booster inoculation of 4 ml of antigen diluted 20 times in sterile physiological saline was administered intravenously to each rabbit 3 weeks after the last subcutaneous inoculation. The rabbits were bled 12 days after the booster inoculation. Two rabbits (Nos 074, 075) inoculated previously with Mycoplasma hyorhinis by the intravenous route were used for subcutaneous inoculation with this antigen. Two rabbits (Nos 104, 105) which had received no previous antigen were used for the subcutaneous inoculation of Mycoplasma granularum (S16).

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[1]Difco Laboratories, Detroit, Michigan.

Specimens from Infected Pigs

Mycoplasma granularum (S16)

Specimens for immunofluorescence were collected from 6 five-month-old, specific-pathogen-free, colostrum-deprived pigs and from one five-month-old conventional pig, which had been infected experimentally. They were inoculated intravenously with 2 ml of a 28 to 30 hour broth culture of the S16 strain which had been passaged 8 to 12 times in artificial medium and cloned 3 times by terminal dilution. The inocula used yielded terminal dilutions in fluid medium of 10^{-8} to 10^{-9}. Specimens were taken from affected joints and popliteal lymph nodes of pigs sacrificed 7 to 10 days post inoculation. Two drops of synovial fluid were placed 3 cm apart on glass slides and allowed to dry at room temperature. The specimens were then fixed for 10 minutes in absolute methanol and, after air drying, the spots were ringed with a Marktex paint pen[1] and stored at 4°C for up to 5 weeks before testing. Suitable pieces of lymph node or affected synovial membrane were placed in OCT compound[2] in small plastic containers cut from serological plates[3] and frozen in previously prepared depressions in solid carbon dioxide. The frozen specimens were wrapped in aluminum foil and stored at -70°C in screw cap bottles. Synovial membrane and lymph node

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[1]Mark-Tex Corp., Englewood, New Jersey.

[2]Ames Co., Elkhart, Indiana.

[3]Model FB48; Linbro Chemical Co., Inc., New Haven, Connecticut.

sections for use in the immunofluorescence test were cut at 4 μm with a model CTI International cryostat[1]. Two consecutive sections from each tissue were placed 3 cm apart on a glass slide and allowed to air dry. The specimens were then fixed for 10 minutes in absolute methanol or in acetone at -20°C for 1 hour, ringed with a Marktex paint pen and stored at 4°C for up to 4 days.

Mycoplasma hyorhinis

Tissues were collected from 9 six-week-old pigs inoculated intraperitoneally with 3 to 4 ml of a 24 to 48 hour broth culture of Mycoplasma hyorhinis (SK76) which had been passaged 8 to 18 times in artificial medium and cloned 3 times by terminal dilution. The inocula had dilution titers of 10^{-8}.

Pigs were sacrificed 6 to 10 days post inoculation and tissues were collected as described previously from affected hearts, lungs, spleens, livers and joints. Impression smears were made from affected heart and lung surfaces and smears were made from pericardial fluid and synovial fluid from affected joints.

Control specimens

Suitable tissues were collected as described above from a 5-month-old, specific-pathogen-free, colostrum-deprived pig in-

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[1]International Equipment Co., Needham Heights, Massachusetts.

oculated intravenously with 2 ml of complete PPLO medium and one conventional 6-week-old pig inoculated intraperitoneally with 3 ml of complete beef heart infusion medium.

Suitable duplicate specimens were taken in all cases for the isolation of mycoplasma in suitable media and for the isolation of bacteria on horse blood agar[1].

Electrophoresis and Serological Techniques

Electrophoresis of fluorochrome conjugated proteins was carried out in polyacrylamide gel with a Buchler polyanalyst[2] at 2.5 ma per tube for two hours[3]. An anionic system with a pH of 9.3 was used according to the instructions of the manufacturers.

Latex agglutination was performed as described by Morton (120). Mycoplasma granularum (S16) antigen was diluted in sterile physiological saline to obtain an optical density of 0.4 in a Coleman Junior spectrophotometer[4] at 420 nm. Antibody to the S16 strain was measured according to a metabolic inhibition test described by Purcell et al. (133).

The indirect fluorescent antibody test was done with immune rabbit sera according to the procedure of Cherry et al. (26).

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[1]Tryptose blood agar base; Difco Laboratories, Detroit, Michigan.

[2]Buchler Instruments, Fort Lee, New Jersey.

[3]These procedures were carried out by Mrs Judith A. Karmon.

[4]Coleman Instrument Corp., Maywood, Illinois.

Fluorescein isothiocyanate labeled anti-rabbit globulin[1] was used at a 1:16 dilution.

Cell Cultures

Cultures of a cell strain derived from synovial fluid of a 3-week-old pig were obtained from M. L. Frey. The cells were initially propagated in a commercial medium consisting of Eagle's basal salts in Earle's saline[2] with 10 per cent fetal calf serum[2], but for cell maintenance the serum content was reduced to 2 per cent. The cell sheets were grown on coverglasses in 35 mm petri dishes containing 1.5 ml medium and infected at 3 days of age with 0.1 ml of a 10^{-2} dilution of a 24 to 36 hour broth culture of Mycoplasma hyorhinis or Mycoplasma granularum (S16). Similarly diluted material from Mycoplasma hyorhinis infected cell cultures were used in some trials and in another trial 0.1 ml of an undiluted 24 hour broth culture of strain S16 was used.

An infected cell culture was examined by immunofluorescence at 1 through 7 days post inoculation to study the sequential development of the mycoplasmas. The cell sheets on the coverglasses were dried at room temperature and fixed with absolute methanol at room temperature for 10 minutes. The coverglasses were broken into 2 or 3 pieces to provide duplicate control

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[1]Lot #806104; Baltimore Biologic Laboratories, Cockeysville, Maryland.

[2]Grand Island Biological Co., New York, New York.

specimens and were either examined immediately or stored for up to 5 weeks at 4°C.

Preparation of Fluorochrome Conjugated Proteins

Reagents

The following reagents were used in the conjugation of fluorescein isothiocyanate (FITC)[1] to immunoglobulins:

1. Saturated ammonium sulfate solution. Enough $(NH_4)_2SO_4$ was added to deionized water at room temperature to obtain a saturated solution. The solution was cooled to 4°C and the pH adjusted to 7.2 with NH_4OH.
2. Saturated solution of $BaCl_2$ in deionized water.
3. Stable biuret reagent[2].
4. 0.5M carbonate-bicarbonate buffer. The buffer was prepared by mixing 50 volumes of 0.5M $NaHCO_3$ and 4.5 volumes of 0.5M Na_2CO_3 so that the pH was 9.0 when the mixture was diluted 10 times.
5. Physiological saline. Deionized water was added to 8.5 g NaCl to make 1 liter and the pH of the solution was adjusted to 7.2 with 0.5M $NaHCO_3$.
6. pH 7.5 phosphate buffered saline (PBS). The buffer consisted of 8.5 g NaCl, 8.5 ml 1M Na_2HPO_4 and 1.5 ml 1M NaH_2PO_4 made up to 1 liter with deionized water.

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[1]Baltimore Biological Laboratories, Cockeysville, Maryland.

[2]Hycel, Inc., Houston, Texas.

7. Dowex 2-X[1], 20 to 50 mesh anion exchange resin in the chloride form.

Purification of globulins

All reagents were chilled to 4°C and procedures were carried out at 4°C. Saturated ammonium sulfate was added to serum on a magnetic stirrer in a dropwise manner to obtain a 40 per cent saturation of the salt and stirring was continued for 6 to 12 hours. The precipitate was sedimented at 8,800xG in a refrigerated angle head centrifuge for 10 minutes. The supernatant was discarded and the sediment was redissolved in enough physiological saline to make the solution one half the original serum volume. The precipitation process was repeated up to 3 times to remove all traces of hemoglobin. The final protein solution was then dialyzed against 20 volumes of physiological saline until all traces of ammonium sulfate had disappeared from the dialysate as determined by lack of precipitate formation when equal volumes of the dialysate and saturated $BaCl_2$ were mixed.

Protein content of the solution was determined with a stable biuret reagent according to the directions of the manufacturers[2]. A Coleman Junior spectrophotometer[3] was used and a standard curve was plotted from values of optical density ob-

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[1]Bio-Rad Laboratories, Richmond, California.

[2]Hycel Inc., Houston, Texas.

[3]Coleman Instruments, Inc., Maywood, Illinois.

tained with dilutions of Lab-Trol standard reference protein[1].

Conjugation

Fluorescein isothiocyanate (FITC) was conjugated to protein in a 1:40 ratio. All reagents were chilled to 4°C and procedures were carried out at 4°C. One volume of a carbonate-bicarbonate buffer was added to nine volumes of the protein solution. FITC was dissolved separately in sufficient carbonate-bicarbonate buffer, previously diluted 1:10, so that when this solution was added to the buffered protein solution the final protein concentration was 1 per cent (w/v). The FITC solution was slowly added to the protein solution on a magnetic stirrer and stirred for 12 hours. The conjugate was dialyzed for 48 hours against 20 volumes of PBS (pH 7.5) containing 10 to 15 g of anion exchange resin and stored at -70°C in 2 or 4 ml aliquots in screw cap glass tubes.

Chromatography

Reagents

1. Type 20 diethylaminoethylcellulose (DEAE cellulose) with a capacity of 0.90 m eq per g[2].
2. 1N NaOH prepared with deionized water.
3. 1N HCl prepared with deionized water.
4. 0.0175M phosphate buffers with pH values of 7.4, 7.2,

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[1]Dade Reagents, Inc., Miami, Florida.

[2]Schleicher and Schuell, Inc., Keene, New Hampshire.

7.0, 6.8, 6.6, 6.4 and 6.2. The buffers were prepared by slowly adding 0.0175M Na_2HPO_4 to 0.0175M NaH_2PO_4 on a magnetic stirrer until the desired pH was obtained.

5. 0.02M pH 6.8 phosphate buffer. The buffer was prepared by slowly adding 0.02M Na_2HPO_4 to 0.02M NaH_2PO_4 on a magnetic stirrer until a pH of 6.8 was obtained.
6. 0.1M pH 6.8 phosphate buffer. 52.6 ml of 1M Na_2HPO_4 and 47.4 ml of 1M NaH_2PO_4 were mixed and made up to 1 liter with deionized water.
7. 0.0175M pH 6.3 phosphate buffer. Deionized water was added to a mixture of 14.6 ml of 1M NaH_2PO_4 and 2.9 ml of 1M Na_2HPO_4 to make 1 liter.
8. 0.05M pH 6.3 phosphate buffer. Deionized water was added to a mixture of 39.4 ml of 1M NaH_2PO_4 and 10.6 ml of 1M Na_2HPO_4 to make 1 liter.
9. 0.1M pH 6.3 phosphate buffer. Deionized water was added to a mixture of 75.2 ml of 1M NaH_2PO_4 and 24.8 ml of 1M Na_2HPO_4 to make 1 liter.

Activation and preparation of DEAE cellulose

DEAE cellulose activation was initiated by stirring 15 g of cellulose into 1 liter of 0.1N HCl in a 2 liter beaker. After standing for 30 minutes, the supernatant was decanted and 1 liter of deionized water was added to the sediment. The supernatant was decanted again after 30 minutes and 1 liter of 0.1N NaOH was added to the slurry which was allowed to sediment

once more. The supernatant was decanted and 1 liter of deionized water was added to the beaker and again allowed to stand for 30 minutes. After removing this supernatant the entire process was repeated two more times. This was followed by several washes of the slurry with deionized water until the supernatant was neutral. The slurry was suspended in deionized water and stored in a beaker at 4°C for up to 4 weeks. The activated cellulose was washed several times with the desired buffer solution and allowed to stand for at least 12 hours in this buffer. It was packed by gravity at 4°C in 1 cm x 20 cm columns and washed with several column volumes of the buffer. Care was taken not to let the columns run dry.

Experimental procedures

Protein-FITC conjugates were dialyzed 24 hours against 20 volumes of the equilibration buffer before they were applied to the columns. Elution was judged by visual tracing of the dye and eluates were collected in test tubes as they emerged from the columns. All elution procedures were carried out at 4°C. Eluates were tested for potency with broth culture antigens and for non-specific fluorescence in swine tissue sections.

Two ml amounts of swine protein-FITC conjugates were carefully applied to DEAE cellulose columns equilibrated with a 0.0175M pH 7.4 buffer. Conjugates were eluted by stepwise reduction of the pH, 0.2 unit per step with at least 10 column volumes of the appropriate 0.0175M buffer. The pH of the eluate was measured to determine the pH at which elution took place.

The elution of 4 ml amounts of swine protein-FITC conjugates equilibrated with 0.1M and 0.02M pH 6.8 phosphate buffers was compared.

Rabbit protein-FITC conjugates were subjected to DEAE cellulose treatment in a manner similar to that of McDevitt et al. (113). The elution of 4 ml amounts of conjugate from columns equilibrated with 0.0175M, 0.05M and 0.1M buffers at pH 6.3 were compared. Eluates obtained with 0.1M buffers at pH 6.3 and 6.8 were also compared.

Absorption of Conjugates

Liver powder absorption

Swine liver powder was prepared by homogenizing 250 g of normal pig liver in 250 ml of chilled deionized water in a Waring blender[1]. One liter of chilled acetone was added to the homogenate on a magnetic stirrer and after stirring for 5 minutes this material was centrifuged at 3,000xG for 10 minutes in a refrigerated angle head centrifuge. The sediment was resuspended in cold PBS (pH 7.5) and centrifuged as before until the supernatant was clear. The suspension was sieved through a double layer of gauze. One liter of chilled acetone was added to the filtrate and the mixture was centrifuged as before. The precipitate was resuspended in chilled acetone (4°C) and centrifuged again. This was repeated three times and the extract was

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[1]Waring Products Corp., New York, New York.

filtered through a Whatman glass filter pad[1] in a Buchner funnel. The material on the filter was washed several times with chilled acetone and dried in a porcelain bowl at 37°C. The powder was stored at 4°C in a dark air-tight container.

Prior to absorption the powder was moistened by suspending 100 to 300 mg in 4 to 5 ml of PBS (pH 7.5) and resedimented in a refrigerated angle head centrifuge at 26,000xG for 10 minutes. The moistened liver powder was then mixed with the FITC conjugate at the rate of 100 mg of dry powder per ml of conjugate, the suspension was incubated for 2 hours at room temperature and centrifuged as before. The supernatant was carefully collected and filtered through a 220 nm cellulose acetate filter[2]. A commercially produced chicken liver powder[3] was used for absorption in the same way.

Absorption with antigens

FITC-protein conjugates were absorbed with the mycoplasma antigens and Escherichia coli cells in attempts to improve their specificity or in the case of homologous absorption to serve as control conjugates. The sediment from one ml of antigen or 0.25 ml of packed E. coli cells obtained by centrifugation in an angle head refrigerated centrifuge at 26,400xG for 20 minutes, was used for absorbing 1 to 2 ml of conjugate. The antigens

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[1]W. R. Balston Ltd., London, England.

[2]Gelman Instrument Co., Ann Arbor, Michigan.

[3]Difco Laboratories, Detroit, Michigan.

were uniformly dispersed in the conjugate with a 2 ml syringe fitted with an 18 gauge canula and the suspension was left at room temperature for 2 hours. The cell suspensions were centrifuged as before and the supernatant was carefully collected and filtered through a 220 nm cellulose acetate filter[1].

Absorption with medium and swine serum

Normal swine serum was used for conjugate absorption in a procedure similar to that of Nilsson (122). Serum was concentrated 3 times either by lyophilization with subsequent reconstitution with distilled water or by pervaporation to one third the original volume. Concentrated serum was added to an equal volume of a FITC-protein conjugate, left 2 hours at room temperature, and then stored at 4°C. Fresh beef heart infusion medium with turkey serum was concentrated and used in the same manner.

Absorption with swine leukocytes

One hundred and twenty ml of swine blood was collected with 20 potassium oxalate tablets[2] dissolved in 5 ml of distilled water and centrifuged in a swing-out head centrifuge at 3,400xG for 30 minutes. The buffy coat was collected with a syringe fitted with a 14 gauge canula. The cells were washed twice with physiological saline using a refrigerated angle head centrifuge

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[1]Gelman Instrument Co., Ann Arbor, Michigan.

[2]Cambridge Chemical Products, Inc., Dearborn, Michigan.

at 8,800xG for 10 minutes to sediment the cells. One fourth ml of packed washed leukocytes was dispersed in 1 ml of conjugate, allowed to react at room temperature for 2 hours and centrifuged as before. The supernate was carefully collected and stored at $4^{o}C$.

Immunofluorescence Procedures

Reagents

1. PBS (pH 7.5).
2. A chelated azo dye counterstain[1]. The counterstain was prepared by dissolving 7.8 mg of eriochrome black T in 10 ml of N,N dimethyl formamide and while the solution was being stirred on a magnetic stirrer a chelating agent was added very slowly. The chelating agent consisted of 50 ml of N,N dimethyl formamide, 20 ml of distilled water, 10 ml of 0.1M $AlCl_3$ and 10 ml of 0.1M acetic acid, adjusted to pH 5.2 with 1N NaOH and made up to 100 ml with deionized water. The counterstain was both prepared and stored at $4^{o}C$ and used for up to one week.
3. Mounting medium. The medium was prepared by mixing 9 volumes of glycerine and 1 volume of PBS (pH 7.5).

Preparation of specimens

One drop of a FITC-protein conjugate was placed on each specimen and spread evenly over an area previously outlined

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[1]Hartman-Leddon Co., Philadelphia, Pennsylvania.

with a Marktex paint pen. The slides were placed on applicator sticks in a covered petri dish containing moist filter paper and incubated for 30 minutes at 37°C. The conjugate was poured off the slides and they were washed in two changes of PBS for 20 minutes and in one change of deionized water for two minutes.

When counterstain was used it was applied before the slides were allowed to dry. The slides were immersed in the counterstain for 30 seconds, washed 2 minutes in 2 changes of PBS, washed 1 minute in deionized water and allowed to dry at room temperature.

Coverglasses were mounted on the slides after applying a small drop of mounting medium on each outlined area. In some cases, specimens were treated with a drop of normal rabbit serum concentrated 3 times by pervaporation. The slides were incubated, washed and dried as described above, before the conjugate was applied.

Cell cultures on coverglasses were treated and washed individually in the original petri dishes and inverted onto a drop of mounting medium on glass slides.

Microscopy

Fluorescence microscopy was achieved with a binocular Leitz Ortholux microscope[1] equipped with a dark field condensor and an Osram HBO 200 mercury vapor lamp. A blue excitatory filter

[1]Ernst Leitz, Wetzlar, Germany.

(BG12) and a yellow barrier filter (K530) recommended by Cherry et al. (26) for bacterial systems was employed throughout this work. A Leitz Mikas micro-attachment with a M1 camera attachment was employed for photography. Photographs were taken on Kodak High Speed Ektachrome film and the ESP-1 process[1] was included in the development procedure.

Titration of conjugates

The intensity of fluorescence was rated from 1+ through 4+. Twofold dilutions of conjugates were made in PBS and each dilution was tested with the homologous broth culture antigen. The highest dilution of conjugate giving a 4+ reaction was used for testing of broth culture antigens but for use with swine specimens the penultimate dilution was used.

Controls

In all cases duplicate specimens were treated with the same conjugate which had been absorbed with homologous antigen and in most cases duplicate specimens were reacted with a heterologous conjugate. Normal swine tissues and uninoculated cell cultures were tested with the conjugates used for swine specimens and cell cultures respectively.

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[1]Kodak Processing Laboratories, Chicago, Illinois.

RESULTS

Titration of Antisera

Latex agglutination

Antisera to the S16 mycoplasma strain, produced by intravenous hyperimmunization of animals, were tested by the latex agglutination test (table 4). Chicken sera consistently exhibited a prozone phenomenon. Agglutination was not detected with preinoculation sera at any dilution. No significant difference was seen between titers of sera collected after 6 inoculations and those taken after the booster inoculation.

Table 4. Reciprocals of the highest dilutions of antisera to strain S16 in which agglutination was detected

Animal	Titer
Rabbit 055	320
Rabbit 056	640
Rooster 2425	320
Rooster 1651	320
Rooster 1650	640
Pig 4840B	320

Metabolic inhibition

Relatively high concentrations of antibody to strain S16 were detected by metabolic inhibition in antisera from the in-

Table 5. Reciprocals of the highest dilutions of antisera inhibiting metabolism by strain S16

Animal	Serum taken before booster inoculation	Serum taken after booster inoculation
Rabbit 055	2496	1248
Rabbit 056	4996	2496
Rooster 2425	N.D.[a]	0
Rooster 1651	0	N.D.
Rooster 1650	N.D.	8
Turkey 775	0	64
Turkey 4275	8	64
Pig 4084B	312	64

[a]Not done.

travenously immunized rabbits and pig, but lower titers were found in chicken and turkey sera (table 5). Preinoculation sera did not inhibit metabolism by this organism at any dilution.

Indirect fluorescent antibody

Sera from rabbits immunized intravenously with Mycoplasma hyorhinis and strain S16 were tested by indirect immunofluorescence to determine the highest twofold dilution exhibiting 2+ fluorescence with homologous broth culture antigens. Sera of rabbits 055 and 056 immunized with strain S16 had dilution

titers of 1/128 and 1/256 respectively and sera from rabbits 074 and 075 immunized with Mycoplasma hyorhinis had titers of 1/640. Sera taken before and after the booster inoculation had similar dilution titers. Fluorescence was not observed with preinoculation sera, but strain SK76 and strain S16 immune sera exhibited fluorescence at dilutions of 1/16 to 1/32 when tested with the heterologous antigen.

Autofluorescence

Broth culture preparations of Mycoplasma laidlawii A, Mycoplasma laidlawii B and non sterol-requiring strains of Mycoplasma granularum (BTS39, 31B-1, Friend, SD7) exhibited yellowish fluorescence with both anti-Mycoplasma hyorhinis and anti-strain S16 conjugates. However, untreated, duplicate specimens exhibited similar fluorescence. The autofluorescence was replaced by a red fluorescence when the counterstain was applied (figure 3). This allowed a clear distinction between immuno- and autofluorescence when mycoplasma strains exhibiting autofluorescence were included in comparison trials (tables 11 and 12).

Medium Component Antigens

Fluorescence with heterologous broth culture antigens was observed when anti-mycoplasma globulin-FITC conjugates were tested for specificity (tables 6 and 7). This non-specific fluorescence was reduced by absorption of conjugates with Mycoplasma laidlawii, concentrated medium and concentrated swine

Table 6. The effect of counterstaining and *Mycoplasma laidlawii* absorption on the specificity of anti-strain S16 conjugate

Mycoplasma antigen	No CS[a] Unabsorbed	CS used Unabsorbed	No CS Absorbed	CS used Absorbed
M. granularum S16	4+[b]	3-4+	4+	3-4+
M. hyorhinis SK76	2-3+	1-2+	2+	-
M. gallisepticum	2+	1-2+	2+	-
M. laidlawii A	2-3+	1+	2+	-
M. fermentans	2+	1-2+	1-2+	-

[a]Counterstain.

[b]Intensity of fluorescence was rated 1+ through 4+.

Table 7. The effect of counterstaining and *Mycoplasma laidlawii* absorption on the specificity of anti-*Mycoplasma hyorhinis* conjugate

Mycoplasma antigen	No CS[a] Unabsorbed	CS used Unabsorbed	No CS Absorbed	CS used Absorbed
M. hyorhinis SK76	4+	3+	4+	3-4+
M. granularum S16	2+	1-2+	1-2+	-
M. laidlawii A	3+	1+	2+	-
M. gallisepticum	2+	1-2+	2+	-

[a]Counterstain.

serum, but not by absorption with E. coli, swine liver powder or chicken liver powder. Absorption with Mycoplasma laidlawii was superior to absorption with medium or swine serum, but absorption alone did not eliminate non-specific fluorescence. Use of both counterstain and conjugate absorption with Mycoplasma laidlawii eliminated non-specific fluorescence almost completely. Use of counterstain alone was inadequate (tables 6 and 7). The counterstain not only reduced non-specific fluorescence, but had a slight quenching effect on specific fluorescence as well. Later it was found that treatment of conjugates with DEAE cellulose eliminated the necessity of using a counterstain, but did not eliminate the necessity of absorption with Mycoplasma laidlawii. In contrast, Mycoplasma gallisepticum grown in beef heart infusion with turkey serum exhibited fluorescence when tested with DEAE cellulose treated anti-strain S16 and anti-Mycoplasma hyorhinis conjugates, but no fluorescence was observed with either conjugate when the organism was grown in FM_4 medium with rabbit serum.

Chromatography

Elution of swine globulin-FITC conjugates

Swine globulin-FITC conjugates eluted from DEAE cellulose columns at pH 6.8 when 0.0175M phosphate buffers were used, but this eluate was very pale and exhibited poor reactivity (1-2+) with homologous antigen. Similar results were observed with eluates of a 0.02M buffer (pH 6.8), but excellent fluorescence

was observed with eluates using 0.1M buffer at pH 6.8. Acrylamide electrophoresis demonstrated that raw conjugates contained the full complement of globulins and some albumin, but eluates from DEAE cellulose columns consisted only of gamma globulin (figure 1).

Elution of rabbit globulin-FITC conjugates

Rabbit globulin-FITC conjugates eluted from DEAE cellulose columns at pH 6.3 with 0.0175M, 0.05M and 0.1M phosphate buffers. However, eluates obtained with 0.0175M and 0.05M buffers were less reactive with homologous antigen (1-2+ and 2+ respectively) than eluates obtained with the 0.1M buffer (4+). Acrylamide electrophoresis revealed that raw conjugates contained all the globulins and some albumin while eluates from DEAE cellulose consisted principally of gamma globulin with traces of beta and alpha globulins (figure 1).

A considerable amount of precipitate developed in conjugates during dialysis against all pH 6.3 buffers prior to treatment with DEAE cellulose. This precipitate was partially soluble in PBS (pH 7.5) and was found by electrophoresis to consist of gamma, beta and alpha globulins (figure 1). However, the rabbit globulin conjugates did not develop precipitates during dialysis against 0.1M phosphate buffer at pH 6.8. Eluates obtained from DEAE cellulose with this buffer exhibited excellent fluorescence (table 8) and contained only gamma globulin (figure 1).

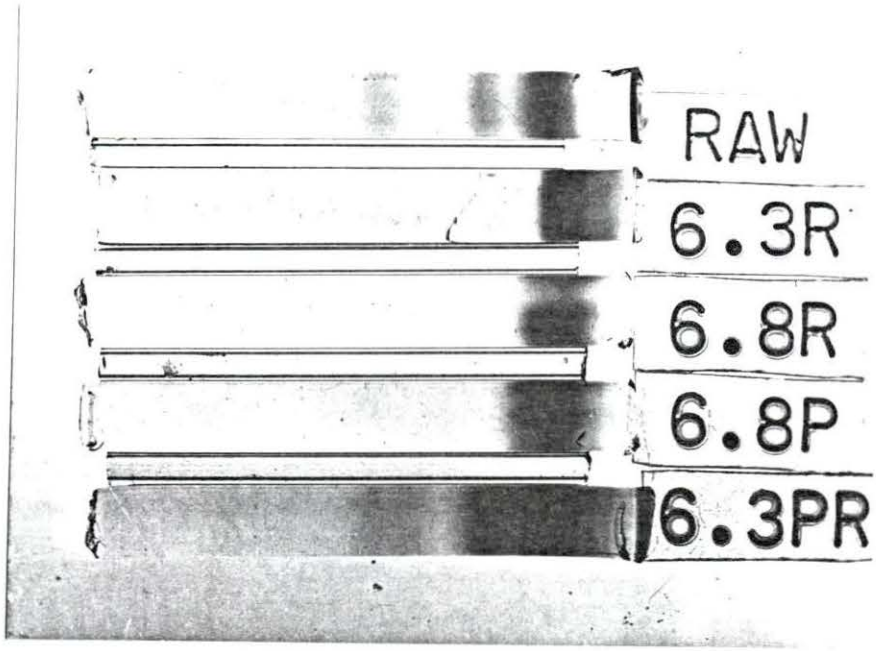
Table 8. Comparison of rabbit anti-*Mycoplasma hyorhinis* globulin-FITC conjugate eluates obtained from DEAE cellulose with 0.1M buffer at pH values of 6.3 and 6.8

Dilution of eluate	Eluate obtained at pH 6.3	Eluate obtained at pH 6.8
Undiluted	4+	4+
1/2	4+	4+
1/4	4+	4+
1/8	3-4+	4+
1/16	3+	3+
1/32	2+	2-3+
1/64	1-2+	1-2+

Specificity of Conjugates with Swine Tissues

Recognition of specific green fluorescence in swine tissues was difficult because of non-specific fluorescence. Use of a chelated azo dye counterstain resulted in an overwhelming red background. A moderate reduction of non-specific fluorescence was observed after absorption of the conjugates with swine liver powder, swine serum and swine leukocytes but not with *Mycoplasma laidlawii* antigen. Pretreatment of specimens with normal rabbit serum resulted in a slight reduction of non-specific fluorescence when rabbit globulin conjugates were used. Treatment of both rabbit and swine globulin conjugates with

Figure 1. Polyacrylamide electrophoresis of FITC-protein conjugates. RAW: rabbit globulin before chromatography; note the presence of all serum proteins. 6.3R: rabbit globulin conjugate after elution from DEAE cellulose with a 0.1M pH 6.3 buffer; it consists of gamma globulin with traces of beta and alpha globulins. 6.8R: rabbit globulin conjugate eluted with 0.1M pH 6.8 buffer; only gamma globulin is present. 6.8P: swine gamma globulin conjugate, eluted with 0.1M pH 6.8 buffer; only gamma globulin is present. 6.3PR: electrophoresis of precipitate which developed in rabbit globulin conjugate during dialysis with 0.1M pH 6.3 buffer; gamma globulin and several other serum proteins are present


RAW
6.3R
6.8R
6.8P
6.3PR

DEAE cellulose markedly reduced non-specific fluorescence except for some moderately bright fluorescence of leukocytes which was partially eliminated by absorption of the conjugates with swine liver powder subsequent to chromatography.

The filtration of conjugates after absorption procedures, especially with homologous antigen, was found to be essential because of fluorescent aggregates which apparently adhered to the specimens.

Comparison of Conjugates

Reactivity of conjugates prepared from various sera was determined by testing twofold dilutions with homologous antigens. The conjugates were all absorbed with Mycoplasma laidlawii and all were treated with DEAE cellulose except those prepared from sera obtained from rabbits and roosters immunized intravenously. A chelated azo dye counterstain was used with the conjugates which were not treated with DEAE cellulose.

Sera from rabbits immunized subcutaneously (SC) were superior to antisera from rabbits and other species immunized intravenously (IV) (tables 9 and 10). Swine serum (IV) was superior to rabbit and chicken sera (IV) (tables 9 and 10).

Comparison of Mycoplasmas by Direct Immunofluorescence

Swine globulin-FITC conjugates and rabbit globulin (SC) FITC conjugates were used for comparison of mycoplasma species by immunofluorescence. Conjugates were treated with DEAE cellulose, absorbed with Mycoplasma laidlawii antigen and, when

Table 9. Comparison of anti-strain S16 conjugates prepared from sera of various hyperimmunized animals

Animal	Method of inoculation	Highest conjugate dilution giving maximum fluorescence
Rabbit 056	Intravenous	1/2-1/4
Chickens 2425 and 1651	"	1/2
Pig 4084B	"	1/4
Rabbits 104 and 105	Subcutaneous	1/8

Table 10. Comparison of anti-Mycoplasma hyorhinis conjugates prepared from sera of various hyperimmunized animals

Animal	Method of inoculation	Highest conjugate dilution giving maximum fluorescence
Rabbit 074	Intravenous	1/4
Pig 4721 and 4722	"	1/4-1/8
Rabbit 074 and 075	Subcutaneous	1/8-1/16

autofluorescing strains were included, treated with a counter-stain.

Non sterol-requiring mycoplasmas did not show a relationship to Mycoplasma hyorhinis (table 12) or to the S16 serotype (table 11). The brightest fluorescence was obtained with

Table 11. Comparison of certain mycoplasmas, including non sterol-requiring strains using anti-S16 conjugate

Mycoplasma	Without counterstain	With counterstain
M. granularum BTS 39	1-2+	-
Friend	2+	-
31B-1	1-2+	-
SD7	2+	-
Strain S16	4+	3-4+
M. laidlawii A	1+	-
M. laidlawii B	2+	-
M. hyorhinis SK76	-	-
M. gallisepticum	-	-
M. fermentans	-	-

homologous antigen (figure 2) when the anti-strain S16 conjugates were employed (table 13). Less intense fluorescence was observed with serologically related strains but fluorescence was not observed with the other mycoplasmas tested. All strains of Mycoplasma hyorhinis exhibited bright fluorescence (figure 4) with anti-strain SK76 conjugates, but fluorescence was not encountered with other mycoplasmas (table 13).

Table 12. Comparison of certain mycoplasmas, including non sterol-requiring strains using anti-*Mycoplasma hyorhinis* conjugate

Mycoplasma		Without counterstain	With counterstain
M. hyorhinis	SK76	4+	3-4+
M. granularum	BTS 39	1-2+	-
	SD7	2+	-
	31B-1	1-2+	-
	Friend	2+	-
Strain S16		-	-
M. laidlawii A		1+	-
M. laidlawii B		2+	-
M. gallisepticum		-	-
M. fermentans		-	-

Cell Cultures

Mycoplasma hyorhinis

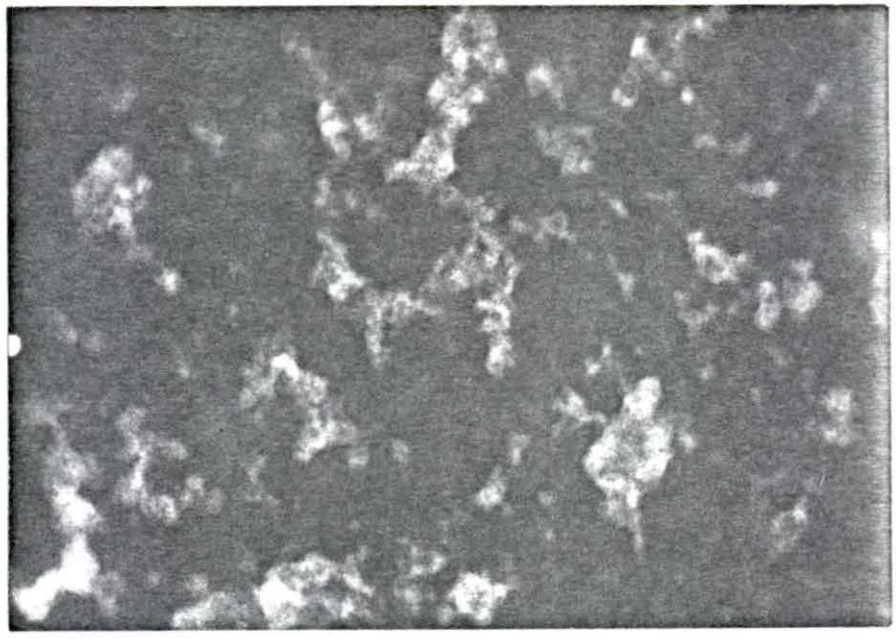
The sequential development of *Mycoplasma hyorhinis* in swine synovial cell cultures was followed up to 4 to 5 days when cell detachment occurred due to a severe cytopathic effect. At 24 hours post inoculation small, brightly fluorescing, pleomorphic bodies with projecting filaments of various lengths, were observed intercellularly and in association with cells (figures 5 and 6). Occasional larger, roughly spherical structures were also seen. Fewer filamentous shapes were discernable at 48

Table 13. Comparison of mycoplasmas by direct immunofluorescence

Mycoplasma	Anti-strain SK76 conjugate	Anti-strain S16 conjugate
Strain S16	-	4+
S149	-	2+
S10	-	2+
33R	-	1+
1918	-	2+
2130	-	2+
3480	-	2+
43L	-	3+
Gubser	-	3+
Jenette	-	3+
Baldwin	-	1+
74BRR	-	1+
M. hyorhinis SK76	4+	-
BTS7	4+	-
GDL	4+	-
FS3	4+	-
T7	4+	-
69N	4+	-
31BRL	4+	-
36BRL	4+	-
85R	4+	-
M. arginini	-	-
M. arthritidis	-	-
M. hyopneumoniae	-	-
M. meleagridis	-	-
M. iners	-	-
M. gallisepticum	-	-
M. synoviae	-	-
M. gallinarum	-	-

Figure 2. M. granularum (strain S16) broth culture sediment smear. Note specific fluorescence of large aggregates of the organism. X1870

Figure 3. M. granularum (strain BTS39) broth culture sediment smear. Specific fluorescence is not present. The organism has taken up the chelated azo dye counterstain resulting in red fluorescence. X1870



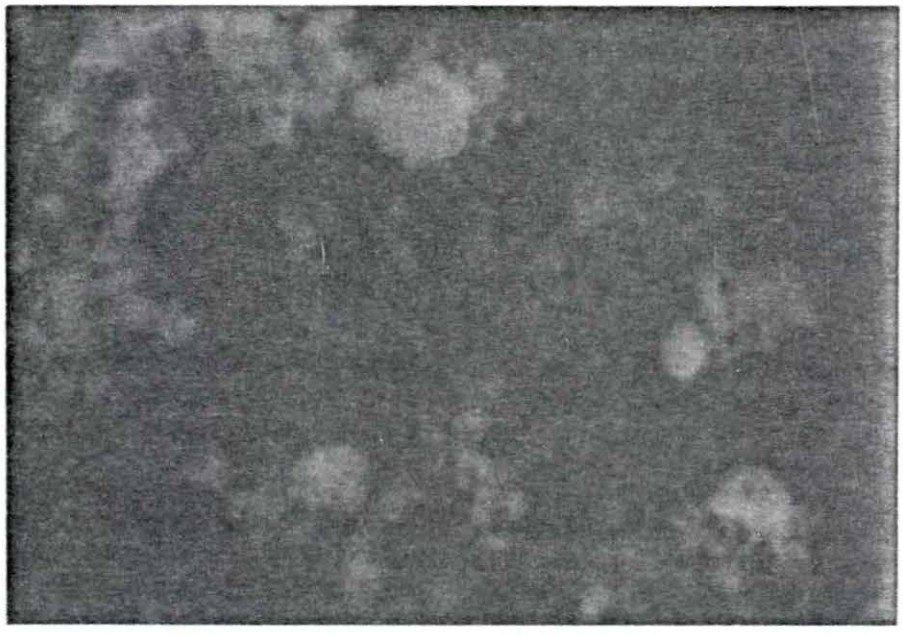
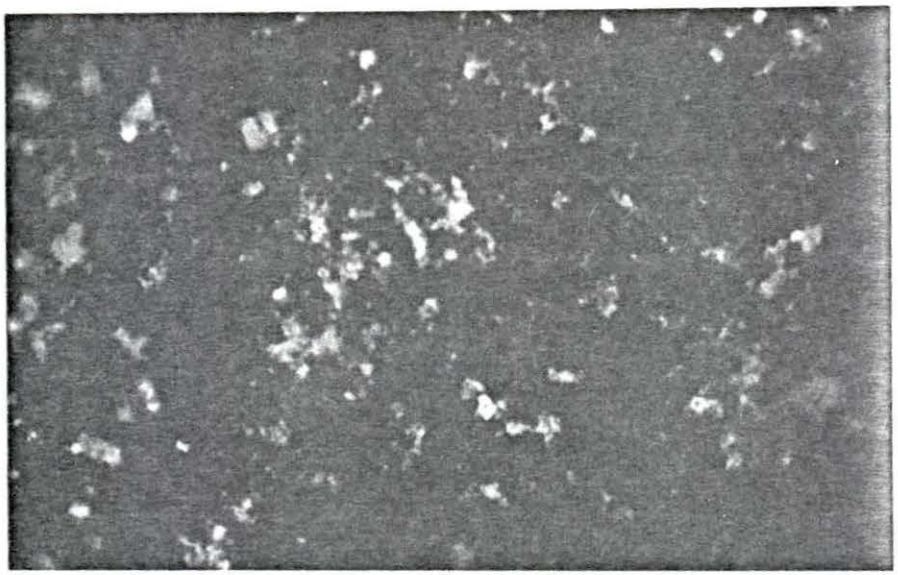
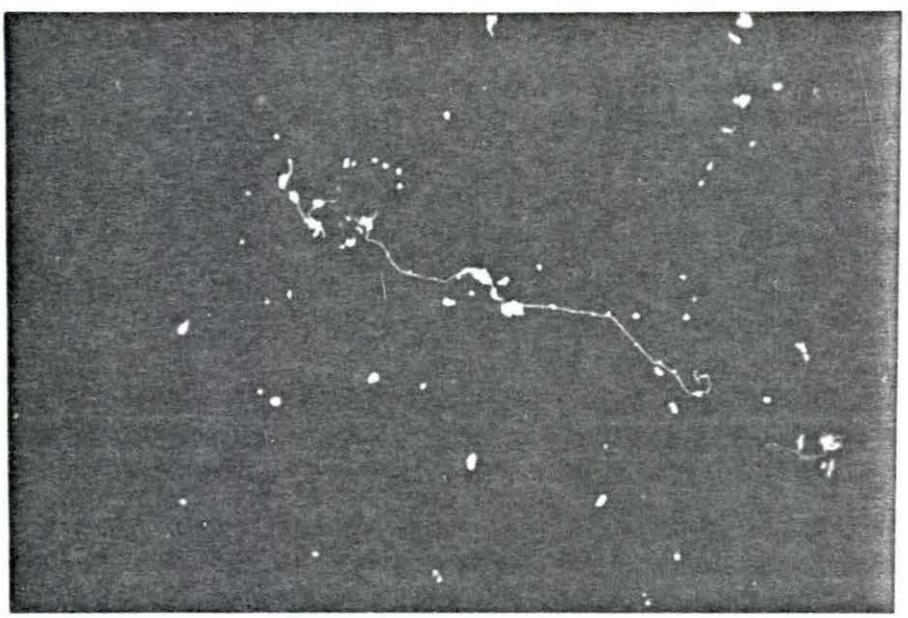


Figure 4. M. hyorhinis (strain SK76) broth culture sediment smear. Aggregates of the organism can be seen but the clumps are smaller than those of M. granularum (strain S16). X1870

Figure 5. M. hyorhinis (SK76) infection of swine synovial cell cultures at 24 hours post inoculation. Note the pronounced filaments and the spherical structures of various sizes. X1870





hours postinoculation, but numerous, larger almost spherical forms of various sizes were observed, some of which appeared to be subdivided to form a faint network. Most of the structures were associated with cells at this stage (figures 7 and 8). At 3 and 4 days post inoculation relatively large spherical structures of a uniform size predominated which were almost entirely cell associated. However, it could not be established whether these structures occurred intracellularly or on the surface of the cells (figure 9).

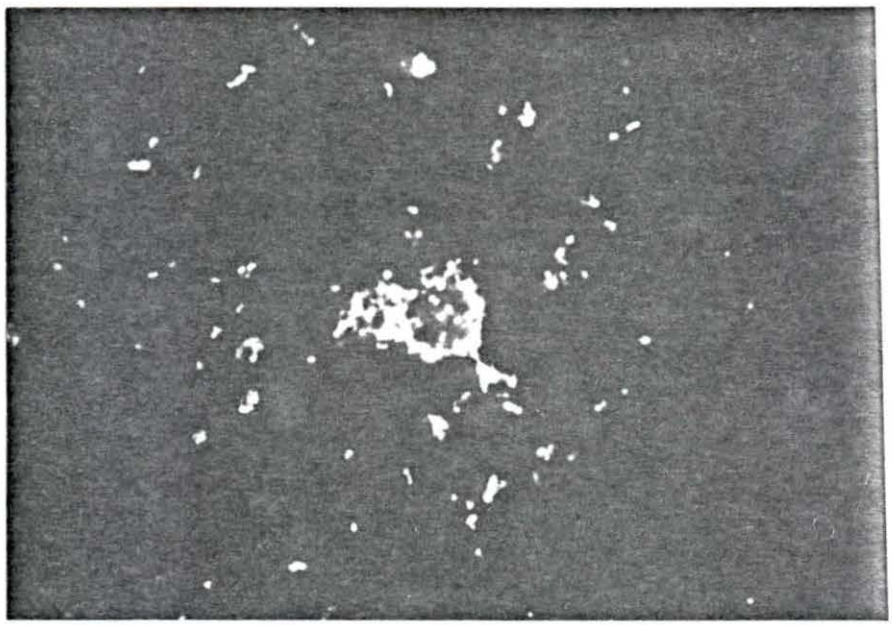
Mycoplasma granularum strain S16

In one trial, a high multiplicity of infection was used which resulted in development of a cytopathic effect in the swine synovial cells within 36 hours. Immunofluorescence demonstrated that the organism was intimately associated with the cell membranes forming a bright outline of the cells either as a uniform layer or as a series of small aggregates along the cytoplasmic membrane (figure 10).

A cytopathic effect did not develop in cells infected with smaller dosages but small pleomorphic cocci and larger irregular aggregates were demonstrated by immunofluorescence at 24 hours post inoculation. These structures exhibited poor fluorescence and very little fluorescence was observed at 3, 4 and 5 days post inoculation. However, a few foci with similar appearing structures were occasionally seen on the 6th and 7th days post inoculation. This fluorescent material was both

Figure 6. M. hyorhinis (SK76) infection of swine synovial cell cultures at 24 hours post inoculation. Note the dense accumulation of pleomorphic shapes in or on a cell. Short filaments project from many individual organisms. X1870

Figure 7. M. hyorhinis (SK76) infection of swine synovial cell cultures at 48 hours post inoculation. Distinct spherical forms of various sizes occur on the surface of several cells. A few similar structures can be seen intercellularly. Filaments are absent. X1870



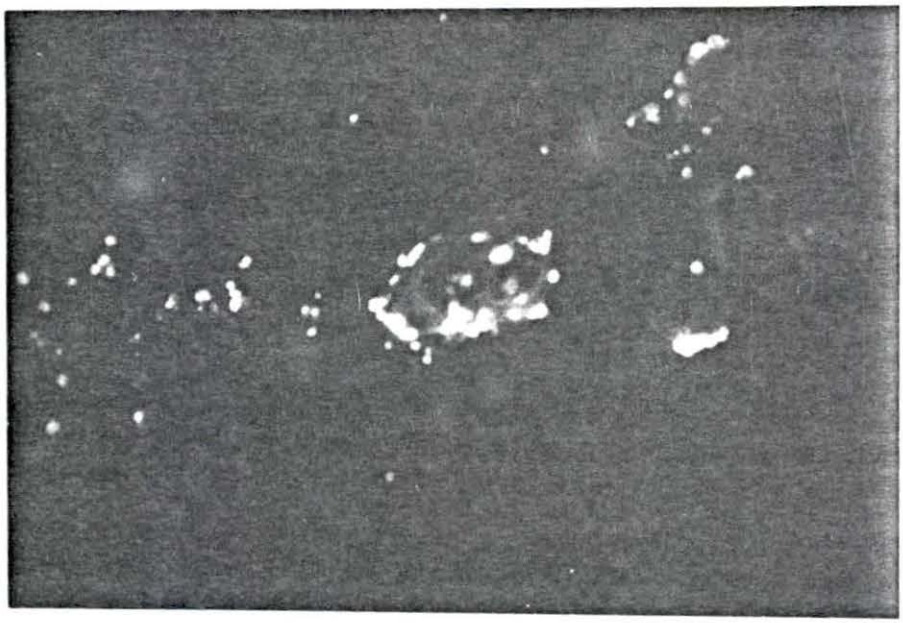
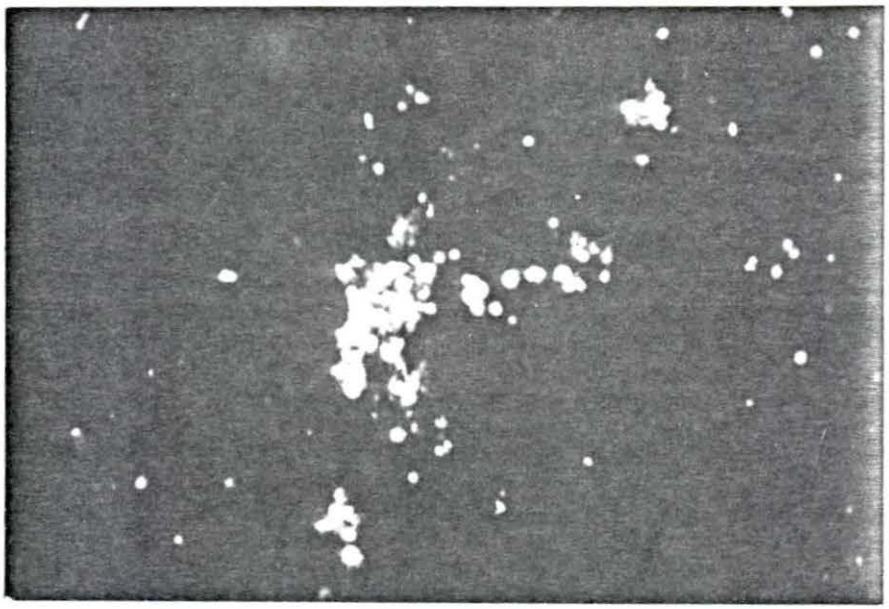
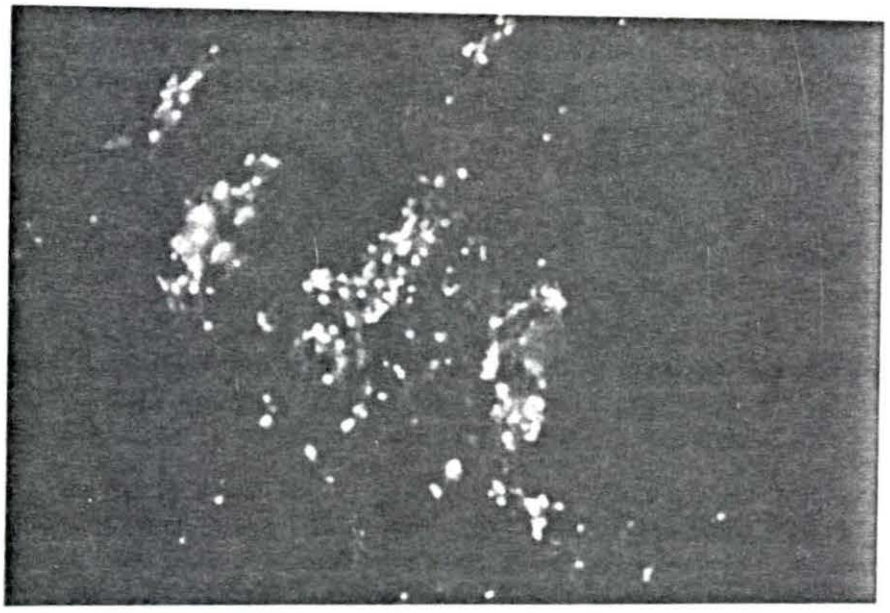


Figure 8. M. hyorhinis (SK76) infection of swine synovial cells at 48 hours post inoculation. Note the complete envelopment of a cell by numerous spherical bodies of various sizes. Many similar forms occur intercellularly. Some of the larger structures appear to be subdivided. X1870

Figure 9. M. hyorhinis (SK76) infection of swine synovial cells at 72 hours post inoculation. Numerous, fairly uniform, spherical, cell associated structures can be seen. X1870





intercellular and cell associated.

Swine Specimens

Conjugates used were hyperimmune swine (IV) and rabbit (SC) serum treated with DEAE cellulose and absorbed with swine liver powder.

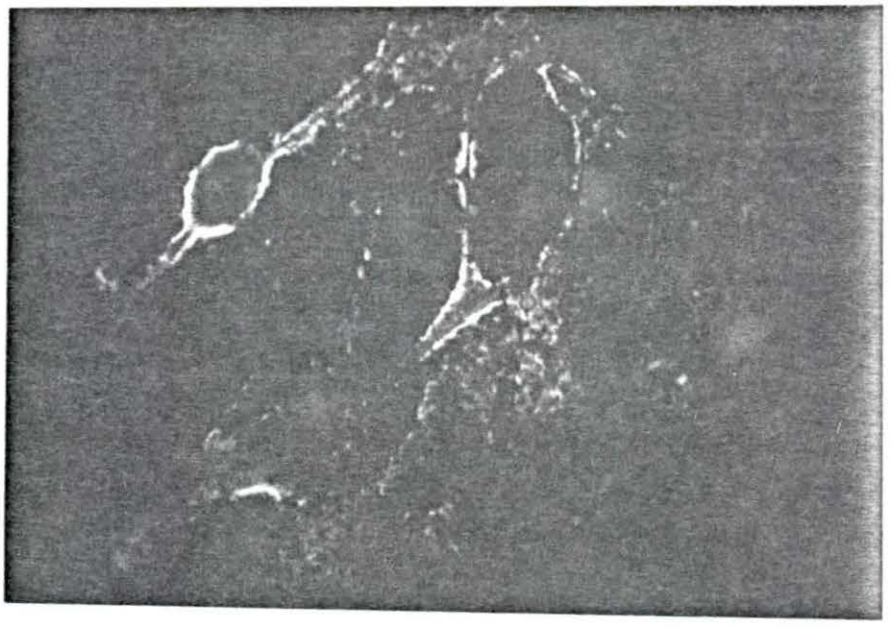
Mycoplasma hyorhinis

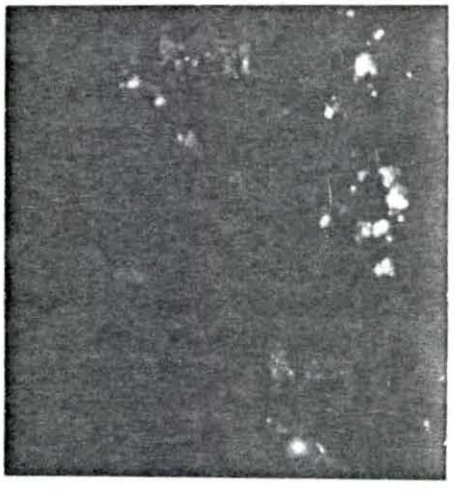
Mycoplasma hyorhinis was detected in serosal exudate in frozen sections of heart, pericardium, liver and spleen, in pericardial fluid smears and in impression smears of heart, pericardium and lung of affected pigs. Scattered small ovoid bodies and larger aggregates were observed intercellularly and many larger, round to pleomorphic structures occurred in association with cells (figures 11, 12 and 13). The organism was detected in sections of 9 of 15 synovial membranes and 15 of 41 synovial fluid smears examined. Small coccoid bodies and larger aggregates were observed mainly in the surface layers of the synovial membranes, but these were often sparsely distributed and frequently occurred in localized foci (figure 14). Occasional fibrin plugs contained large numbers of the organism. Mycoplasma hyorhinis was isolated in beef heart infusion medium from all the joints sampled for immunofluorescence.

Fixation with methanol resulted in some quenching of fluorescence by the organism. Fluorescence was not observed in duplicate specimens stained with conjugates absorbed with homologous antigen or with anti-strain S16 conjugates.

Figure 10. M. granularum (S16) in swine synovial cell cultures at 24 hours after a high multiplicity infection. Note the heavy concentration of fluorescent staining along the cell surfaces. X1870

Figure 11. Pericardium section from a M. hyorhinis (SK76) infected pig. Irregular, roughly spherical forms can be seen in localized areas in the exudate and they are apparently associated with cells. X13500





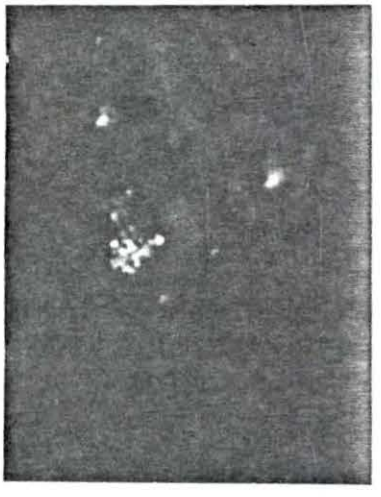
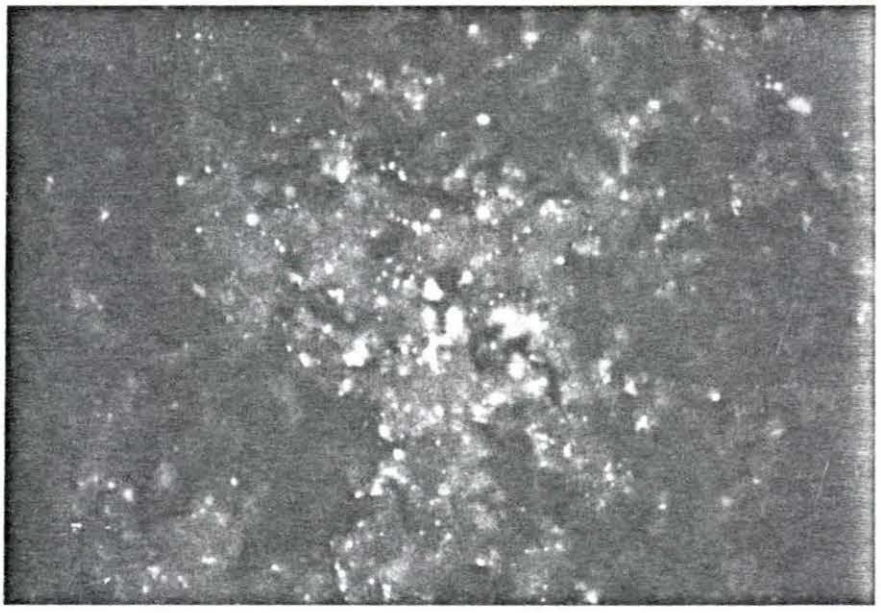
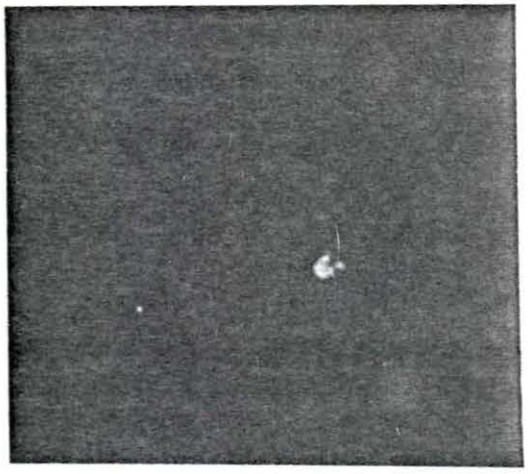
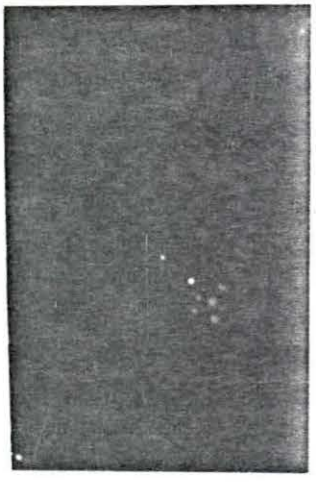


Figure 12. Pericardium section from a M. hyorhinis (SK76) infected pig. Numerous fluorescent intercellular cocci and coccal aggregates can be seen. X1870

Figure 13. Impression smear of the lung from a M. hyorhinis (SK76) infected pig. Note the spherical forms in or on several cells. X1620







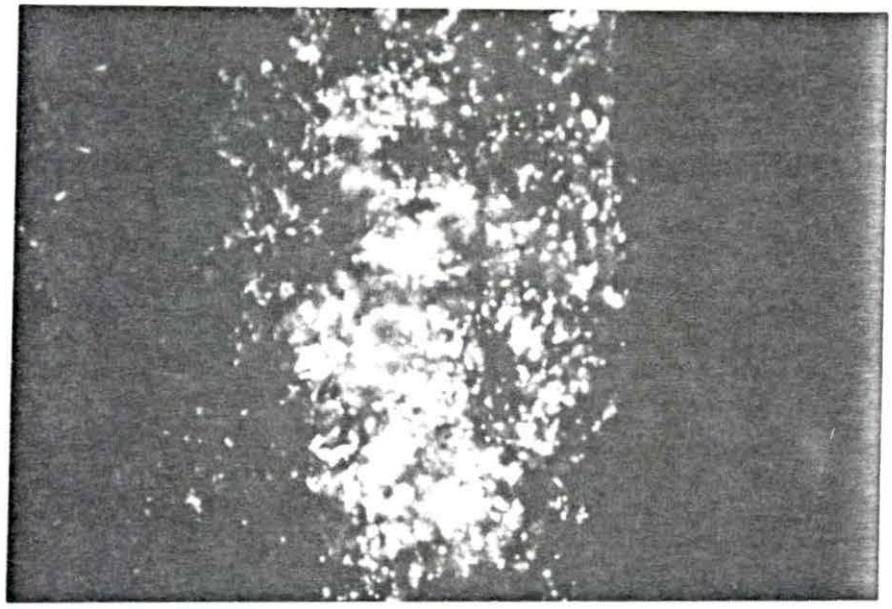
Mycoplasma granularum strain S16

Examination of synovial membranes from arthritic joints of pigs experimentally infected with strain S16 revealed a filamentous network of specific fluorescence in the subsynovial tissue (figures 15, 16 and 17). In places this network was apparently associated with the cytoplasmic membranes of cells. Isolated foci of fluorescent filaments occurred closer to the synovial surface (figure 15). Some filaments and small coccal aggregates were observed in the surface layers of membranes but these were sparse and occurred irregularly (figures 18 and 19). The organism was detected in sections of all of 11 synovial membranes and in 14 of 35 synovial fluid smears originating from joints which had yielded the organism. Filamentous structures were observed in 2 of 6 lymph nodes examined.

Fluorescence was not observed in normal synovial membranes treated with anti-strain S16 conjugates, nor in infected specimens with anti-*Mycoplasma hyorhinis* conjugate or with anti-strain S16 conjugate absorbed with homologous antigen.

Figure 14. Section of a synovial membrane from an arthritic joint of a M. hyorhinis (SK76) infected pig. Note the numerous coccal aggregates in and immediately below the superficial cell layers of the membrane. X1870

Figure 15. Section of a synovial membrane from an arthritic joint of a M. granularum (S16) infected pig. Note the foci of fluorescent filaments in the subsynovial area. X865



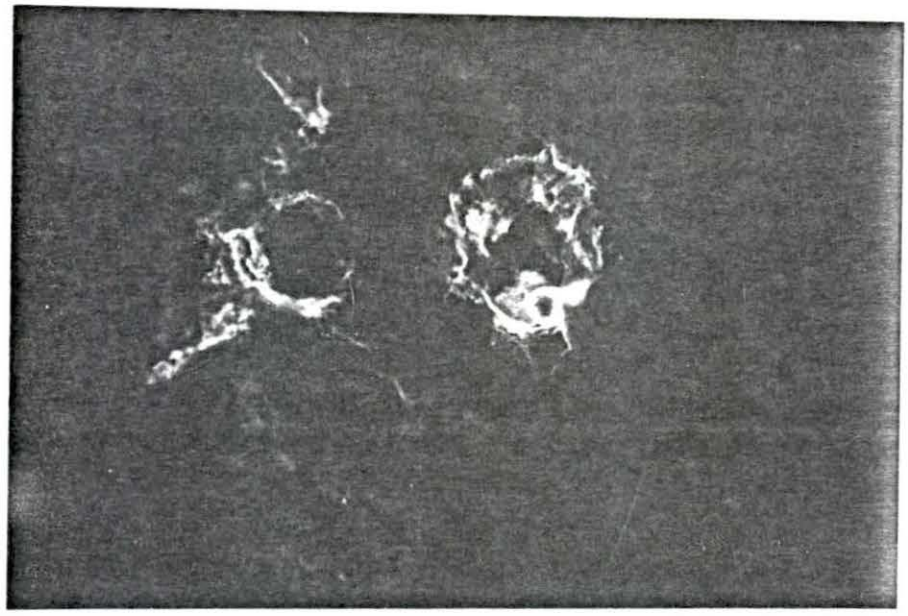
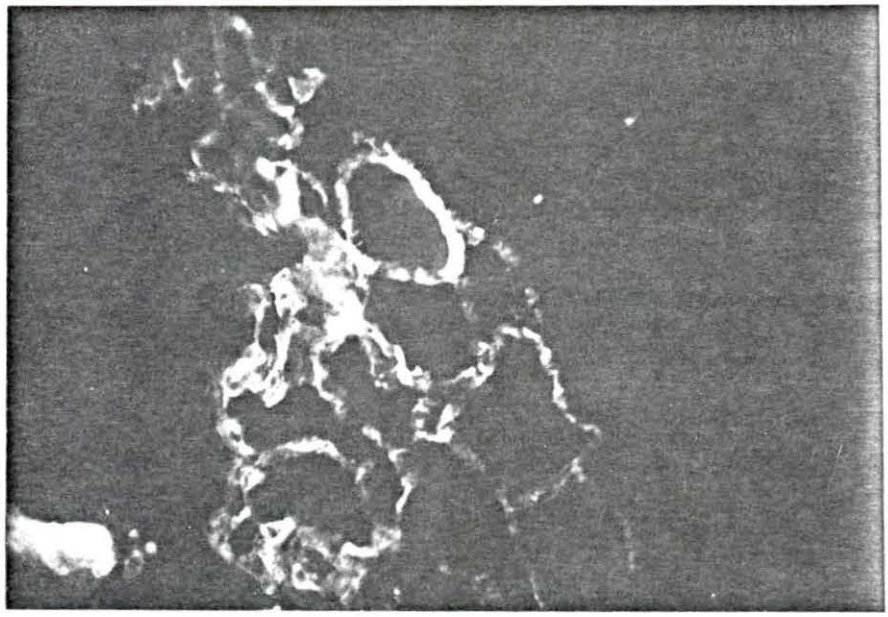


Figure 16. Section of a synovial membrane from an arthritic joint of a M. granularum (S16) infected pig. Note the network of fluorescent filaments. X865

Figure 17. Section of a synovial membrane from an arthritic joint of a M. granularum (S16) infected pig. Note the network of fluorescent filaments. X1870



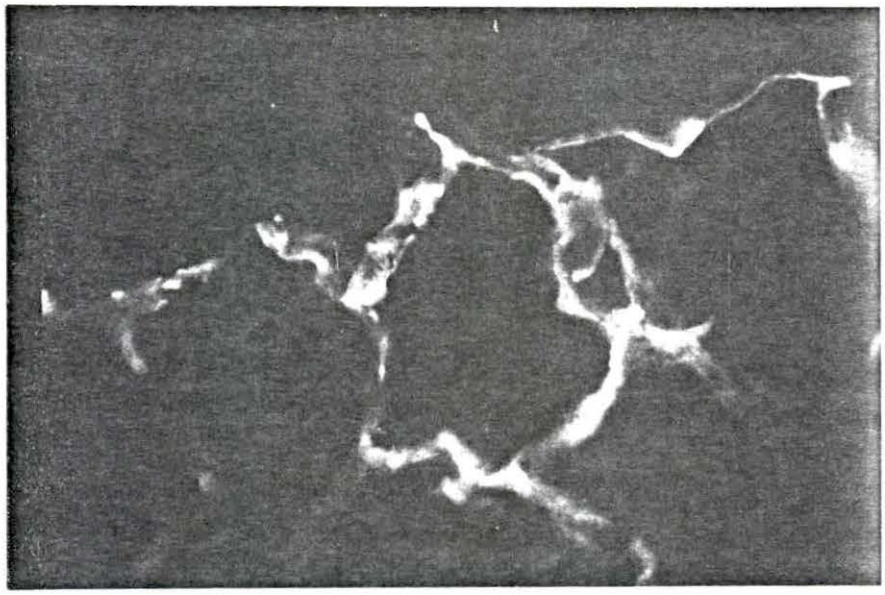
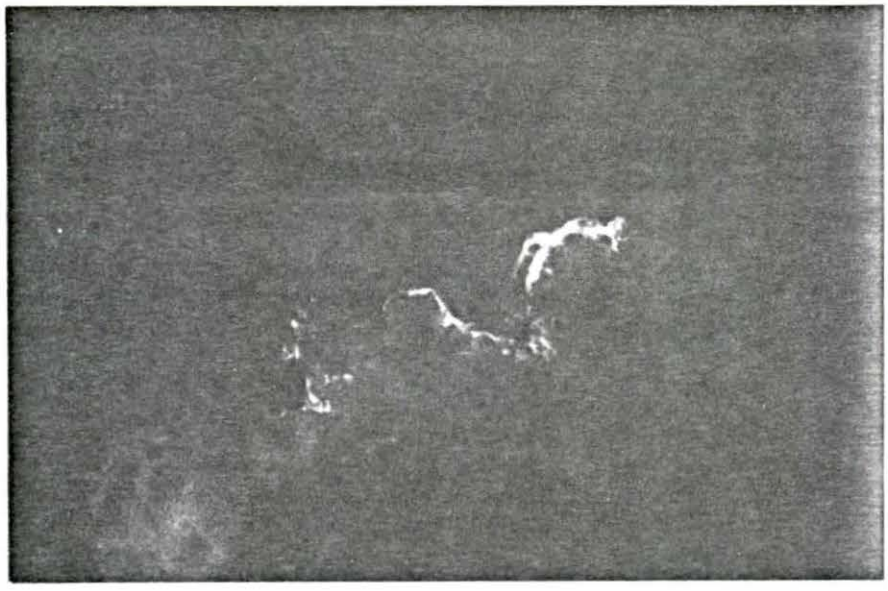
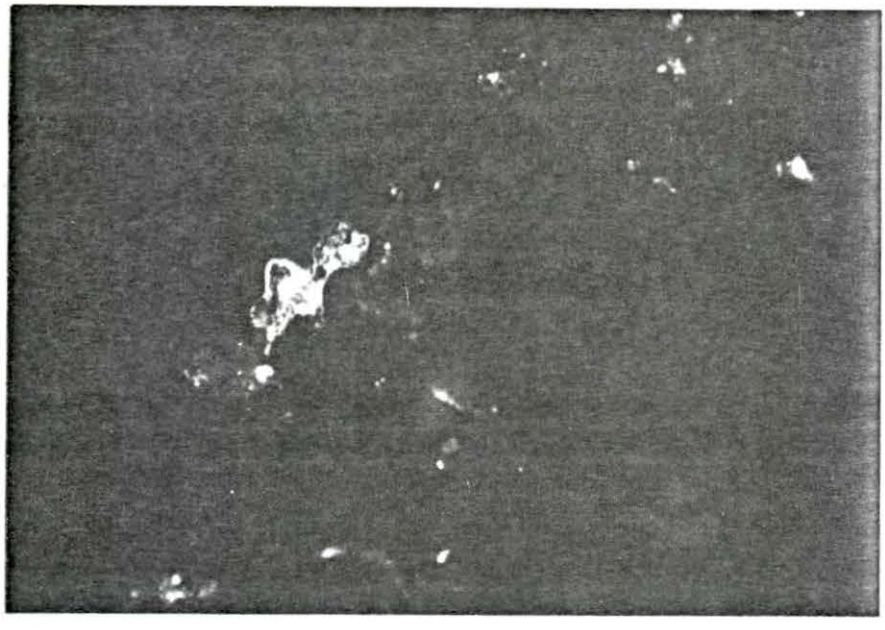


Figure 18. Section of a synovial membrane from an arthritic joint of a M. granularum (S16) infected pig. Note the filaments on the surface and in between cells of the superficial cell layers. X1870

Figure 19. Section of a synovial membrane from an arthritic joint of a M. granularum (S16) infected pig. Note filaments and coccal aggregates on the surface and in between the superficial cell layers. X1870





DISCUSSION

Broth Culture Antigens

Satisfactory specific immunofluorescence with cultures of Mycoplasma hyorhinis and Mycoplasma granularum was achieved after consideration of 3 factors: autofluorescence, antibody to medium components and non-specific fluorescence. Each of these problems was initially treated independently and their ultimate solutions required separate procedural measures.

Yellowish autofluorescence was observed with the non sterol-requiring mycoplasmas. This autofluorescence was replaced by a background fluorescence when a chelated azo dye counterstain was used. Use of this dye therefore facilitated the recognition of autofluorescence.

Blue autofluorescence of mycoplasma colonies has been described (24, 40, 157), however, the nature of autofluorescence depends to a large extent on the barrier filter used (121). The wavelength of light emitted by the excitatory filter used in this study peaks at about 410 nm with a range of 325 to 475 nm and although the amount of light absorbed by fluorescein increases as the wave length of excitatory light is increased above 400 nm, the contrast between auto- and specific fluorescence becomes less obvious (121).

Another source of error in immunofluorescence of broth culture antigens was attributed to the presence of antibodies to medium components in conjugates. An example of this was that

fluorescence was observed with Mycoplasma gallisepticum grown in beef heart infusion with turkey serum, with both Mycoplasma granularum and Mycoplasma hyorhinis conjugates but not when it was grown in FM_4 medium with rabbit serum. Absorption of conjugates with beef heart infusion medium and especially absorption with Mycoplasma laidlawii grown in this medium reduced fluorescence due to heterologous antigens. Absorption of conjugates with chicken liver powder or use of chelated azo dye counterstain did not significantly reduce non-specific fluorescence due to medium antibodies.

Presence of medium component antibody in mycoplasma (51, 89, 93, 155) and tissue culture cell (69) immune sera has been reported. The contaminating antigens could not be eliminated by washing the preparations (69, 89, 155). Several investigators circumvented this problem by growing the mycoplasmas in medium containing serum from the animal species used for immunization (40, 51, 93). This approach was not attempted in this study because of the number of animal species used and even if it had been used, the problem would probably not be entirely eliminated as antibodies to medium components other than serum proteins have been reported (89).

Non-specific fluorescence is an inherent problem with immunofluorescence and therefore the remarks made here apply also to immunofluorescence with cell cultures and swine specimens. Non-specific fluorescence was reduced slightly by masking with a chelated azo dye counterstain and virtually eliminated

by treating conjugates with DEAE cellulose. The counterstains that have been used with immunofluorescence for the identification of mycoplasmas include chelated azo dyes (15, 123) and albumin conjugates of lissamine-rhodamine RB200 (107) and rhodamine sulfochloride (151). Nairn (121) warned that careful evaluation of controls is required because the distribution of non-specific staining of FITC conjugates and the staining distribution of the counterstain may not coincide.

The most common causes of non-specific fluorescence include the presence of unreacted fluorescein which may persist after dialysis (121), high fluorescein to protein ratios (63, 81, 113, 130) and the presence of conjugated proteins other than gamma globulin in conjugates (121, 136).

Although treatment with DEAE cellulose may result in the loss of most of the macroglobulins and up to 50 per cent of total antibody from conjugates (121), several investigators have successfully used this procedure to reduce non-specific fluorescence (35, 38, 63, 67, 91, 113, 136). DEAE cellulose treatment of conjugates eliminated unreacted fluorescein (121), conjugated gamma globulin molecules with a high fluorescein to protein ratio (63, 113) and conjugated serum proteins other than gamma globulin (35, 113, 136). Buffers of various molarities and pH levels were employed by investigators to equilibrate DEAE cellulose columns (38, 63, 67, 113, 136).

In this study a pH 6.8 0.1M phosphate buffer was found to be suitable for both swine and rabbit globulin conjugates.

McDevitt et al. (113) obtained optimal results with rabbit globulin conjugates after DEAE cellulose treatment with a 0.05M phosphate buffer at pH 6.3, but in this study eluates obtained with a similar buffer exhibited poor fluorescence. However, eluates of rabbit globulin conjugates obtained with a pH 6.3 0.1M buffer exhibited excellent fluorescence with minimal non-specific fluorescence in spite of traces of beta and alpha globulins.

Loss of gamma globulin due to precipitation during dialysis of rabbit globulin conjugates at pH 6.3 prior to DEAE cellulose chromatography apparently did not reduce the potency of the conjugates. Precipitation did not develop to any extent in swine or rabbit globulin conjugates dialyzed against pH 6.8 0.1M phosphate buffer. DEAE cellulose eluates obtained with this buffer contained only gamma globulin and exhibited excellent fluorescence with minimal non-specific staining.

Sterol-requiring strains of Mycoplasma granularum were found to be serologically homogenous by growth inhibition, metabolic inhibition and immunodiffusion (144) but a considerable variation in their staining intensity was observed with immunofluorescence. The brightest fluorescence was obtained with the homologous strain of Mycoplasma granularum, but this strain specificity was not encountered with Mycoplasma hyorhinis strains.

Mycoplasma laidlawii absorption was employed to eliminate medium component antibody from Mycoplasma hyorhinis and Myco-

plasma granularum conjugates. A close serological and physiological relationship between Mycoplasma laidlawii and the prototype non sterol-requiring strain of Mycoplasma granularum (BTS39) has been reported (93, 181). This would seem to indicate that absorption with the former organism of anti-Mycoplasma granularum conjugates would also remove specific antibodies.

However, a sterol-requiring strain (S16) of Mycoplasma granularum was used in this study and recent work has established that non sterol-requiring strains differ serologically and physiologically from sterol-requiring strains (144) which reduces the strength of the argument. The study indicated that strain S16 was not related to the non sterol-requiring strains of the organism but it may be argued that if there were a relationship it would not be observed after absorption with Mycoplasma laidlawii. The object of these studies was not to determine the relationship between these strains but rather to produce specific conjugates.

The previously reported relationship between Mycoplasma hyorhinis strains originating from cell cultures and those of swine origin (132, 156) was confirmed by immunofluorescence.

Swine Tissue Specimens

Several investigators have reported that chelated azo dye counterstains caused little apparent quenching of specific fluorescence (15, 68, 123) but in this study counterstaining

resulted in an overwhelming red background in conjugate treated infected swine tissues and cell cultures. Alternative methods of reducing non-specific fluorescence were thus investigated.

One of the simplest and most effective means of reducing non-specific fluorescence was conjugate dilution (24, 81, 86, 112, 183). Dilution of conjugates used in this study, especially those prepared from sera of animals immunized intravenously, resulted in an excessive loss of potency which was probably due to a low antibody concentration. Low antibody titers often develop in sera of animals immunized with mycoplasmas (35, 50, 53).

Absorption of conjugates with swine liver powder, swine serum or swine leukocytes resulted in a moderate reduction of non-specific fluorescence. Numerous investigators have reported using tissue powders for reduction of non-specific fluorescence (9, 16, 48, 64, 157, 158) but others found that this procedure was of limited value for this purpose (63, 113) and actually reduced conjugate potency (113, 121).

A slight reduction of non-specific fluorescence resulted after pretreatment of specimens with concentrated non-immune rabbit serum when rabbit conjugates were used or after absorption of conjugates with concentrated non-immune swine serum. Nilsson (122) used the latter procedure to eliminate non-specific fluorescence in lymph nodes. Nairn (121) pointed out that the disadvantage of pretreating specimens with non-immune serum was that some exchange occurred between the attached un-

conjugated non-immune and the conjugated immune globulins.

DEAE cellulose treatment of conjugates markedly reduced non-specific staining except for some moderately bright non-specific fluorescence in leukocytes. This was considerably reduced by absorption with swine liver powder. Non-specific fluorescence of leukocytes has frequently been reported (34, 113, 121, 141). It was found to be brightest in fresh unfixed specimens (121) and was not eliminated by DEAE cellulose treatment of conjugates (113). Absorption of conjugates with an anion exchange resin (32) or with tissue powder, especially marrow powder (121), reduced but did not eliminate non-specific leukocyte fluorescence.

DEAE cellulose chromatography reduces the fluorescein to protein ratio of conjugates (63, 113). Tissue powder absorption of conjugates with a low fluorescein to protein ratio is more efficient than absorption of conjugates with a high ratio in reducing non-specific fluorescence. This may explain why greater reduction of leukocyte non-specific fluorescence was obtained with liver powder absorption of conjugates after DEAE cellulose treatment.

The reason why Mycoplasma hyorhinis or Mycoplasma granularum (S16) was not detected by immunofluorescence in a large proportion of synovial fluid samples that were culturally positive is not known and was not fully investigated. A considerable amount of material came off the slides during the washing procedures which may have accounted for some negative results or the con-

centration of the organisms may have been too low for them to be detected by immunofluorescence.

Butler and Leach (14) reported that immunofluorescence was not as sensitive as direct isolation for the detection of low grade mycoplasma infections of cell cultures. The intensity of fluorescence is dependent on the concentration of antibody (121). It is possible that the potency of these particular conjugates may have been inadequate to detect the organisms in synovial fluid. Attempts to improve efficiency of fluorescein conjugates for detection of mycoplasmas in synovial fluid by prolonged immunization were unsuccessful. The rationale of this approach was based on results reported by other workers that they obtained an improved antibody response to mycoplasmas in animals after prolonged immunization (50, 53).

Mycoplasma granularum (S16) was detected in all the affected synovial membrane sections examined but Mycoplasma hyorhinis was not detected in a proportion of membranes infected with that organism. The distribution of the latter organism in synovial membranes was localized and often sparse. This may account for the failure of Duncan and Ross (45) to demonstrate it by electron microscopy.

Morphology

Broth culture antigens of Mycoplasma hyorhinis and Mycoplasma granularum (S16) consisted of aggregates of various sizes with some scattered individual coccoid bodies. The aggregates

and individual organisms of strain S16 were larger than those of Mycoplasma hyorhinis. Ring forms or filaments were not seen in preparations of either organism grown in broth cultures.

Mycoplasma hyorhinis exhibited a transitory filamentous phase in cell cultures which confirms the observations of Freundt made in broth cultures (54). Occasional filaments in cell culture strains of Mycoplasma hyorhinis have been reported (84, 88). Filamentous growth is a common manifestation in the early growth phase of many mycoplasmas (54, 134). Filaments were not observed in Mycoplasma hyorhinis broth culture preparations or in infected swine tissue specimens, but chronological studies were not done in these systems.

After the filamentous phase, large, fairly uniform, spherical objects developed in cell cultures, some of which appeared intercellularly at first but later they became almost entirely cell associated. The structures appeared to be on the surface of the cells but it could not be determined whether some occurred intracellularly or not. Small aggregates and scattered cocci were observed in exudates on visceral organs of pigs infected with Mycoplasma hyorhinis.

Larger round structures, similar to those observed in cell cultures, were noted in association with cells in these exudates but they were not seen in synovial membrane sections. Definite cell association could not be demonstrated in the synovial membrane with this organism. Roberts et al. (140) described bodies resembling Mycoplasma hyorhinis in degenerating mononuclear

cells in the subsynovial spaces, but in this study the organism was not detected to any extent in the subsynovial area.

Mycoplasma granularum (S16) was intimately associated with the cytoplasmic membrane of cells of a synovial cell culture after a high multiplicity infection. The organism formed a uniform fluorescent layer forming an outline of the cells except that along some cytoplasmic processes numerous coccal aggregates were observed attached to the cell surface. A similar observation was made by Clyde (27) with Mycoplasma pneumoniae. Fluorescence was less intense and gradually disappeared when a low infection dose was employed. Occasional foci of moderately bright fluorescence were observed at 5 to 7 days post inoculation but since the study did not extend beyond the 7th day, it was not established whether these foci were manifestations of renewed growth.

The fluorescent network observed in the subsynovial areas of synovial membranes infected with strain S16 was apparently made up of numerous filamentous structures. It may be argued that this was due to the association of the organism with the cytoplasmic membranes of cells as was observed in cell cultures. This bizarre pattern of fluorescence was observed in specimens from two separate experiments. Mycoplasma hyorhinis was detected mainly in the superficial layers of the synovial membrane while strain S16 was not observed in this area except for occasional filaments and coccal aggregates. The morphology of the structures observed in Mycoplasma granularum (S16) infected

synovial membranes seems to resemble, to a certain extent, a strain of Mycoplasma bovigenitalium which was described as large nests of delicate filaments or microtubules (85).

SUMMARY

Fluorescein isothiocyanate conjugates were prepared with sera of various animals hyperimmunized with Mycoplasma hyorhinis (strain SK76) and Mycoplasma granularum (strain S16). The most potent conjugates were prepared from sera of rabbits immunized with a series of subcutaneous injections of whole organism in combination with an adjuvant. Slightly less potent antisera were prepared in swine by intravenous inoculations although they were superior to those prepared in rabbits and chickens by that route.

Non-specific fluorescence was most effectively removed by diethylaminoethylcellulose (DEAE cellulose) chromatography. The optimal buffer pH and molarity for elution of both swine and rabbit globulin conjugates from columns were determined. Non-specific fluorescence of leukocytes in swine tissues and exudates was reduced satisfactorily by absorption of DEAE cellulose treated conjugates with swine liver powder. Antibodies to medium components were removed by conjugate absorption with Mycoplasma laidlawii grown in a suitable medium. Non sterol-requiring mycoplasmas autofluoresced, but this was eliminated with a chelated azo dye counterstain. The conjugates were species specific when tested with a variety of mycoplasmas. The Mycoplasma granularum (S16) conjugates were also somewhat strain specific.

Mycoplasma hyorhinis exhibited a transitory filamentous phase in swine synovial cell cultures but large spherical bodies

predominated in later stages. In these cell cultures Mycoplasma granularum became intimately associated with cytoplasmic membranes of the cells after a high multiplicity infection. Unsatisfactory results were obtained with a lower infection dosage possibly because the organism failed to grow.

Examination of sections of visceral organs from pigs experimentally infected with Mycoplasma hyorhinis revealed cocci and larger spherical bodies in the serosal exudate. Fluorescent coccoid forms were observed in the superficial cell layers of synovial membranes from arthritic joints of these animals. Mycoplasma hyorhinis was detected in 15 of 41 synovial fluid smears made from culturally positive joints.

Synovial membranes and some lymph nodes from pigs experimentally infected with Mycoplasma granularum exhibited a network of fluorescent filaments. Fluorescence was observed mainly in the subsynovial area of the synovial membrane, but small coccal aggregates and filaments were noted irregularly between and on the superficial cell layers. The organism was detected in 14 of 35 synovial fluid smears originating from joints which had yielded the organism.

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ACKNOWLEDGEMENTS

I greatly appreciate Dr. R. F. Ross' assistance during the course of the work and in the preparation of this thesis. The work with cell cultures was done in collaboration with Dr. M. L. Frey.

A special word of appreciation goes to my wife, Carol, who aided me in innumerable ways throughout the course of study and for typing this manuscript.

This investigation was supported in part by U.S. Public Health Service grants AI-AM07921 and AI-AM07922 from the National Institute of Allergy and Infectious Diseases.