COMPARISON OF ISOLATES OF PORCINE MYCOPLASMA HYORHINIS BY INDIRECT HEMAGGLUTINATION

by

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INTRODUCTION

Microorganisms of the genus Mycoplasma are nonmotile, pleomorphic, frequently require serum enrichment for growth in artificial medium, do not produce endospores, stain poorly with ordinary bacterial stains, frequently pass bacteria-retaining filters and form minute raised translucent colonies on solid medium. Parasitic species of these microorganisms are widespread in man and animals. They are frequently isolated from the secretions of the mucous membranes of normal individuals and from certain lesions in the lungs, mammary glands and nasal cavity. Saprophytic species are frequently found in sewage, manure, humus and soil. Mycoplasma is the only genus in the order Mycoplasmatales (18).

Mycoplasma hyorhinis is of widespread occurrence in swine. It is frequently isolated from the nasal cavity, is one of the most common secondary invaders in pneumonia and is the cause of an acute serositis and arthritis. Observations made in this laboratory indicate that detectible variation occurs in the morphology, growth characteristics and pathogenicity of various isolates of this organism. The development of an adequate serological test for detection of antibodies against this organism would aid in evaluation of the antigenic relationship of various isolates, enable assays of the immune response and possibly assist in diagnosis.

Complement fixation, serum neutralization, growth inhibition, agglutination and precipitation tests have been employed to demonstrate antigen-antibody reactions in the case of Mycoplasma hyorhinis. Growth inhibition, agglutination and precipitation were effective only with low dilutions of hyperimmune serum. Although high neutralization indexes were obtained with hyperimmune rabbit and rooster serum the results were not reproducible. Complement fixation has been employed with hyperimmune rabbit serum and serum from experimentally infected swine.

This work was undertaken to develop a suitable serological test for the detection of antibodies against Mycoplasma hyorhinis. Several serological techniques were evaluated. These included neutralization of cytopathic effect in cell culture, growth inhibition, hemagglutination, agar-diffusion precipitation and indirect hemagglutination. Indirect hemagglutination was the only procedure that gave encouraging results in preliminary trials. Therefore, the major portion of this work was directed toward development of a suitable indirect hemagglutination test for use with Mycoplasma hyorhinis. Certain morphological, cultural and pathogenic properties were determined and correlated with the serological results obtained.

REVIEW OF LITERATURE: PART 1. *MYCOPLASMA HYORHINIS*

Mycoplasma hyorhinis is a nonmotile, minute, filter passing, coccoid rod capable of growth in serum enriched artificial medium, chicken embryos and cell cultures. Characteristic minute colonies grow aerobically on suitable solid medium. It is found only in swine. *Mycoplasma hyorhinis* is classified among the pleuropneumonia-like organisms in the order Mycoplasmatales (18).

History

This organism was described and named by Switzer in 1953 (183, 184), 1954 (181, 182, 186) and 1955 (185). A similar agent was reported by McNutt *et al.* in 1945 (117), however, their agent was not propagated in artificial medium and was not identified as a pleuropneumonia-like organism. Additional work with *Mycoplasma hyorhinis* has been carried out by Carter and McKay (30), Carter (25), Willigan and Beamer (200), Cole (42), Ose (151), Switzer (179) and Lecce (108).

Pathogenic Properties

The normal habitat of *Mycoplasma hyorhinis* appears to be the nasal cavities of swine. About 60 percent of 90 swine with grossly normal nasal turbinates were found to have this organism present in their nasal cavities (181). However,

this organism has been established to be the cause of naturally occurring cases of sero-fibrinous pericarditis, pleuritis, peritonitis and arthritis in swine (25, 42, 109, 116, 151, 184, 186, 199). It is frequently isolated from pneumonic swine lungs, but is considered to be a secondary invader (25, 31, 110, 186).

Mycoplasma hyorhinis produces a sero-fibrinous pericarditis, pleuritis, peritonitis and arthritis when inoculated intraperitoneally into pigs up to 6 weeks of age (25, 108, 182, 199). Intranasal instillation or aerosol exposure does not result in detectible lesions (25, 184). An isolate of Mycoplasma hyorhinis of nasal origin was recovered from rectal swabs as long as 21 days after intranasal instillation (188). Mycoplasma hyorhinis is nonpathogenic for chickens, mice, guinea pigs, rabbits, calves and sheep (185).

Propagation of Mycoplasma hyorhinis in Artificial Media, Cell Cultures and Chicken Embryos

This organism can be propagated in suitable fluid medium containing 10 to 20 percent serum (30, 185). Addition of maltose (42, 151), peptone (151) or yeast autolysate* (108) has been found beneficial. Growth occurs in fluid medium as a slight turbidity and fine deposit of sediment (181).

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*Switzer, W. P., Ames, Iowa. Studies on atrophic rhinitis. Private communication. 1959.

Minute colonies develop in 48 to 72 hours on suitable solid medium (25, 185). The colonies are glistening, have regular edges, exhibit little tendency to coalesce and frequently possess a central elevation.

Mycoplasma hyorhinis may be propagated in chicken embryos by yolk sac inoculation (30, 42, 108, 151, 183, 199). About one-half of the embryos inoculated develop lesions or die (185). Mortality begins on the 4th or 5th day after inoculation and may extend as long as 15 days postinoculation (42, 181, 199). Lesions observed in chicken embryos infected with this agent include hyperemia, myocarditis, pericarditis and focal hepatic necrosis (42, 185, 199). The lesions produced depend on the age of the embryo at inoculation and the length of time they survive (42, 185). Six-day-old embryos are more susceptible than older embryos (42).

Mycoplasma hyorhinis can be propagated in serial passage swine kidney, nasal mucosa, lung and endothelium cell cultures (179). It also grows in primary swine kidney and lung cell cultures (179). The cytopathic effect observed in these tissues can be produced on initial isolation or after 100 transfers in artificial medium (179). The organism was also propagated in primary bovine fetal lung, swine lung and chicken embryos but no cytopathic effect was noted (42).

Characteristics of the Organism

Mycoplasma hyorhinis is a coccoid rod measuring from 0.3 to 0.6 microns in length (181, 183, 185, 199). Mycoplasma hyorhinis stains intensely blue black (199) or bluish purple* with Giemsa's stain (185), blue with Macchiavello's stain (185, 199), is faintly Gram negative (185, 199) and is not acid-fast (185, 199). Preparations of Mycoplasma hyorhinis photographed with the electron microscope exhibit irregular flattened spheres with central vacuoles (185) or short coccoid rods with a uniform density (199).

Mycoplasma hyorhinis may be stored at least 10 months at -40°C. or lyophilized and stored at 4°C. for at least a year (185). Organisms in yolk sac fluid from infected embryos were viable after 3 years but not after 7½ years storage at -20°C. (42). This organism will withstand heating at 56°C. for 30 minutes but not for 60 minutes (185).

The organism regularly passes a Selas 02 filter (182, 183) and a few organisms will even pass a Selas 05 filter (181). A sterilizing Seitz filter or a Mandler filter candle of 7 pounds bubbling pressure retained the organism (181).

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*Switzer, W. P., Ames, Iowa. Studies on atrophic rhinitis. Private communication. 1959.

No detectible acid or gas is produced in lactose, sucrose, dextrose, maltose or mannite (185). However, in medium conditioned by prior growth of Escherichia coli, dextrose and maltose are fermented (151).

Electron transfer occurring during growth of Mycoplasma hyorhinis can be detected by reduction of triphenyl tetrazolium (42, 185). Certain strains have been reported to lose this ability and regain it several passages later (42). The organism does not hemolyze sheep erythrocytes or agglutinate horse, chicken, guinea pig, rabbit, sheep or dog erythrocytes (42). It is relatively insensitive to penicillin, bacitracin and thallous acetate (108, 183). It is slightly susceptible to streptomycin (108, 183) and susceptible to aureomycin (108, 183), terramycin (108, 183), erythromycin (187) and chloromycetin (108).

Antigenic Properties

Neutralizing antibodies were not detected in serum from roosters, rabbits or pigs hyperimmunized with Mycoplasma hyorhinis (183). Subsequent neutralization tests indicated that hyperimmune rabbit and rooster serum contained neutralizing antibodies (42, 151). The significance of these findings is difficult to determine because the results were not consistent. Slide agglutination (42), plate agglutination (151), tube agglutination (151) and precipitation (151)

tests were positive when Mycoplasma hyorhinis cell suspensions and hyperimmune rabbit or rooster serum were used. Rabbit and chicken antisera were successfully employed to inhibit growth of Mycoplasma hyorhinis in fluid cultures (151). Complement fixation was successfully employed to detect antibodies in hyperimmune rabbit serum and in serum from pigs experimentally infected with Mycoplasma hyorhinis (151). Subsequent to initiation of this work an unpublished report of a trial of an indirect hemagglutination test with Mycoplasma hyorhinis was found (151).

Serological Tests Employed with Other Mycoplasmataceae

Mycoplasma mycoides antigens have been studied by complement fixation (21, 50, 153, 154), flocculation (153, 154), slide agglutination (147, 148, 154), precipitation (50) and agar diffusion precipitation (198). Subsequent to initiation of this work an indirect hemagglutination test was reported for Mycoplasma mycoides (47).

Mycoplasma of human origin have been studied by growth inhibition (56, 91), tube agglutination (56) and complement fixation (91, 118, 175). Complement fixation (57) and agglutination (57, 83) have been employed with canine mycoplasma. Mycoplasma gallinarum and other avian Mycoplasma have been studied by means of complement fixation (77), precipitation (77), agglutination (1, 2, 3, 77, 84, 90, 94,

95, 202) and growth inhibition (203). Mycoplasma gallinarum will hemagglutinate chicken red blood cells (193). Inhibition of hemagglutination is widely used as a diagnostic test (48, 58, 77, 90, 94, 95, 197). Inconsistent results were obtained when indirect hemagglutination was applied to Mycoplasma gallinarum (94).*

*Yoder, H. A., Ames, Iowa. Studies on Mycoplasma gallinarum. Private communication. 1959.

REVIEW OF LITERATURE: PART 2. INDIRECT HEMAGGLUTINATION

Certain polysaccharide and protein antigens can be adsorbed to properly prepared erythrocytes. Specific antibody will combine with these modified erythrocytes and cause them to agglutinate. This indirect hemagglutination has been utilized for the detection and titration of antigens and antibodies and for the differentiation of closely related antigens.

History

Burnet and Anderson in 1946 demonstrated that erythrocytes modified with an antigen could be agglutinated by specific antiserum (20). They observed that erythrocytes exposed to Newcastle disease virus were agglutinated by specific immune serum. Keogh et al. in 1947 found that a polysaccharide from Hemophilus influenzae could be adsorbed to washed erythrocytes (101). The agglutination of these erythrocytes by specific antiserum from infected patients was used as a diagnostic aid. Erythrocytes with adsorbed antigens of Mycobacterium tuberculosis were shown to be agglutinated by specific antiserum by Middlebrook and Dubos in 1948 (125). This test has been widely studied (52, 66, 70, 78, 81, 120, 121, 156, 159, 168, 169, 194).

Boyden in 1951 demonstrated adsorption of protein antigens to erythrocytes which had been treated with dilute

tannic acid (13). These modified erythrocytes were agglutinated by specific antiserum. This test has found wide application in the study of protein antigens and their antibodies (16, 41, 51, 80, 99, 103, 115, 127, 168). Various terms have been used to describe the agglutination of modified erythrocytes by antiserum. These include 'hemagglutination' (26), 'passive hemagglutination' (8), 'conditioned hemagglutination' (128), 'indirect hemagglutination' (29), 'enterobacterial hemagglutination' (133) and 'sensitized erythrocyte agglutination test' (113). The term indirect hemagglutination has been used most frequently in the last few years (22, 47, 73, 152).

Physical and Chemical Aspects of Indirect Hemagglutination

Sheep erythrocytes are usually employed for indirect hemagglutination (60, 65, 125, 130, 201), however, human (130), dog (130), horse (12), ox (52), goat (46), chicken (164), rabbit (130), guinea pig (130), mouse (201), rat (130) and monkey (201) erythrocytes have been used. Fresh erythrocytes are usually employed but formalin preserved erythrocytes can be used (49, 59, 114, 192). Formalin preserved cells will adsorb polysaccharide antigens and after tannic acid treatment, will adsorb protein antigens.

Electrolytes have been shown to be necessary for the adsorption of polysaccharides by erythrocytes (143, 144).

This adsorption will take place in isotonic NaCl, potassium chloride or sodium citrate solution, but not in 5 percent glucose, 5 percent sucrose or isotonic calcium chloride solutions (140). Serum proteins inhibit the adsorption of antigens to erythrocytes (137, 142). For this reason it is imperative that erythrocytes be washed several times prior to attempting antigen adsorption.

Optimum conditions for adsorption of bacterial polysaccharides to erythrocytes depend on temperature, time, antigen concentration and erythrocyte concentration (130, 201). Erythrocytes will adsorb polysaccharides more rapidly at 37°C. than at lower temperatures (201). The time required for adsorption of polysaccharides to take place varies from 30 minutes to 4 hours (133, 164, 190, 201). The optimum time is dependent on the concentration of antigen employed, since erythrocytes are more rapidly modified by concentrated than by dilute polysaccharide solutions (87, 103, 130). In contrast, protein antigens are as completely adsorbed by tannic acid treated erythrocytes in 10 minutes at 4°C. as at higher temperatures or longer periods of time (13).

It has been demonstrated for several Gram negative bacteria that 2 to 10 micrograms of polysaccharide per milliliter of 1 percent erythrocytes is the minimal amount needed to obtain maximum sensitivity of the erythrocytes to

specific antibody (103). Similar studies utilizing tannic acid treated erythrocytes and protein antigens from Pasteurella pestis revealed that maximum sensitivity of erythrocytes to specific antibody was obtained with 4 micrograms of antigen per milliliter of 2.5 percent erythrocyte suspension (103).

It is believed that each antigen is adsorbed to specific receptors on the erythrocyte. In most cases, several antigens may be adsorbed by erythrocytes with no reduction in agglutinability by each homologous antiserum (130, 133, 136, 163, 164). Several polysaccharide antigens may be adsorbed to erythrocytes consecutively or simultaneously. Tannic acid treated erythrocytes will adsorb both protein and polysaccharide antigens simultaneously or separately (103).

Many of the chemical and physical aspects of indirect hemagglutination were considered by Gard in 1951 (74, pp. 107-109).

> . . . the surface in a physical sense is not always identical with the chemical cell boundary, which in turn may differ from the physiological or the immunological surface. From a chemical point of view, the cell is generally supposed to possess an outer membrane of proteins and lipoids in several layers. . . . The backbone consists of two radially oriented monolayers of lipoids covered on both sides by tangential monolayers of protein, with interpolated patches of non-oriented lipoids. In accordance with this concept, which is based upon permeability studies, chemical analysis has revealed proteins and lipoids as the main constituents of the cell membrane, in the ratio

> of 1.7 to 1. . . . polysaccharides . . . cover about 0.1 percent of the total surface. . . . One might conceive the polysaccharide chain molecules as filamentous, flexible excrescences from the surface, occuring singly or in small tufts. Proteins and polysaccharides are generally hydrophilic. . . . Lipoids, on the other hand, are less rich in polar groups. Often the molecule has one hydrophilic and one hydrophobic end. . . The cell surface, therefore, has to be regarded as a mosaic of alternating hydrophilic and hydrophobic areas. The solubility of the cells, or their capacity to form stable suspensions, will depend upon the ratio of the two types of surface constituents. Antibodies seem to be unable to pass the osmotic barrier at least with their specific activity intact. Consequently only such cellular antigens as are exposed on the surface can react with antibodies in the medium. It is possible, therefore, to speak of an immunologic cell surface as well. Under certain conditions antigens may be non-reactive although exposed. In some cases the relief of the surface, for purely mechanical reasons may not allow contact with the comparatively large antibody molecules. Another cause of non-reactivity, less readily understood, is the so-called steric inhibition. In such cases antigens are mechanically accessible but, on account of the chemical configuration of neighbouring areas, they are more or less completely shielded by electrical and other intermolecular forces. . . . Of particular interest is the high reactivity of the surface proteins which seem to be able to adsorb a large number of different lipoids and polysaccharides. By adsorption of the former class of substances the ratio of hydrophobic to hydrophilic groups will increase, with a diminished solubility and increased tendency to agglutination as a consequence. Adsorption of polysaccharides acts in the opposite direction but will, however, add new antigens to the cell surface, and thus make the cell able to react with antibodies to which previously no affinity was present.

Modification of erythrocytes with polysaccharides results in no change in their ability to react in the agglutination of human types O, A, and B erythrocytes by specific antisera, in lysis of sheep erythrocytes by amboceptor

and complement, in agglutination of rabbit erythrocytes by type A botulinum toxin, in agglutination of chicken erythrocytes by PR-8 influenza virus or in lysis of human type O erythrocytes by alpha toxin of Clostridium welchii (201). Erythrocytes treated with receptor destroying enzyme, periodate or pancreactic lipase were still capable of adsorbing bacterial polysaccharides (201).

Adsorption of polysaccharides by erythrocytes is inhibited by human serum (142), various animal sera (142), egg yolk (142), fractions of rat liver (142), lecithin (15, 137, 146), cholesterol (15, 137, 146), and cephalin (15).

Antigens Employed in Indirect Hemagglutination Tests

Polysaccharide antigens employed in indirect hemagglutination tests are adsorbed from cell suspensions, supernatants of cultures, washed cells or disrupted cells. Solvent extraction is frequently used to concentrate the antigen.

Polysaccharide antigens can be extracted from Mycobacterium tuberculosis by phenol precipitation (125). A fraction of Mycobacterium tuberculosis extracted with this procedure contained 90 to 98 percent polysaccharide (121). Only 40 percent of this polysaccharide could be adsorbed to erythrocytes (121). Phenol insoluble antigens from Hemophilus influenzae (100), Mycobacterium paratuberculosis

(62), Mycobacterium phlei (62), and Pasteurella tularensis (82) have also been successfully used in indirect hemagglutination tests.

Supernatants of heated suspensions of Hemophilus pertussis (63), Escherichia coli (129, 130), Pasteurella multocida (26), Vibrio comma (60), Salmonella typhimurium (164), Salmonella gallinarum (164), Salmonella pullorum (164), and Vibrio fetus (190) contain polysaccharide antigens which can be adsorbed to erythrocytes. The amount of heating required to liberate these antigens varies from 30 minutes at 56°C. for Hemophilus pertussis (63) to 2 hours at 120°C. for Vibrio fetus (190). Other methods employed for the extraction of polysaccharides utilize trichloracetic acid and ethanol (150), formamide (102), ether (34) and sodium taurocholate and alcohol (33).

Boyden in 1951 found that washed erythrocytes treated with dilute tannic acid were capable of adsorbing protein antigens (13). A commercial antigen of Mycobacterium tuberculosis obtained by trichloracetic acid precipitation (tuberculin P.P.D., Weybridge) was adsorbed to tannic acid treated sheep erythrocytes. Protein and polysaccharide antigens in this precipitate were separated by adsorbing the polysaccharide to washed sheep red cells and the protein to tannic acid treated washed sheep red cells (120). Protein antigens from Mycobacterium tuberculosis were also coupled

to formalin preserved erythrocytes with tetrazotized benzidine (43).

Other preparations containing antigens which adsorb to tannic acid treated erythrocytes include suspensions of lyophilized cercariae of Schistosoma mansoni (96), an ammonium sulphate precipitate of sonic treated Hemophilus pertussis (80), an acid soluble fraction of Trichinella spiralis larvae (161), an acid soluble fraction of Toxoplasma gondii (92) and an extract of Ascaris spp. larvae (97).

Applications of Indirect Hemagglutination

Numerous clinical studies using indirect hemagglutination have been carried out on human tuberculosis (44, 45, 66, 75, 156, 159, 160, 167, 168). False reactions are sometimes obtained with this test. In one study the test was fairly reliable in acute tuberculosis but frequently gave false negative reactions in chronic cases (167). Nonspecific reactions were frequent when the indirect hemagglutination test was applied to bovine tuberculosis (52, 64, 81, 194).

Indirect hemagglutination has been used in the detection and titration of antibodies against enteric bacteria. Titers of 1:320 were found in children with dysentery due to Shigella sonnei (136). Antibodies against Escherichia coli

have been detected and titrated in sera from random human populations (138), newborn infants (177, 178), adult volunteers fed enteropathogenic Escherichia coli (178) and baby pigs fed colostral antibody (172).

Indirect hemagglutination is more specific and sensitive than bacterial agglutination in the case of Escherichia coli (104, 126, 138), Pasteurella pestis (166), Aerobacter aerogenes (126), Pseudomonas aeuruginosa (71, 126), Salmonella typhimurium (165), Pasteurella multocida (26), Vibrio fetus (190), Proteus spp. (126), and Paracolobactrum spp. (126). Antibodies against Vibrio fetus in bovine vaginal mucus were detected earlier and with greater sensitivity with indirect hemagglutination than with bacterial agglutination (191). Adsorption techniques indicated that different antibodies react in indirect hemagglutination and bacterial agglutination in the case of Hemophilus pertussis (63) and Klebsiella spp. (155). Several workers have found indirect hemagglutination to be more sensitive and more specific than complement fixation (68, 73, 105). Studies on the antigenic characteristics of a number of bacterial species have been conducted (10, 26, 27, 62, 88, 102, 120, 129, 163, 169, 170).

It has been observed that the simultaneous addition of complement and antibody to modified erythrocytes results in hemolysis (14). This hemolysis usually occurs only with

sheep erythrocytes (14, 64, 65) but hemolysis of protein modified tannic acid treated human erythrocytes has been reported (51). This hemolytic test has been used as a serologic aid in studies of Mycobacterium tuberculosis (14, 51, 62, 64, 123), Escherichia coli (130), Streptococcus spp. (51, 157), Diplococcus pneumoniae (9), Hemophilus pertussis (65) and Shigella dysenteriae (131). A comparison of indirect hemagglutination and the hemolytic test indicated that the latter is superior for the study of enterobacterial O antigens, while the former is superior for the study of Vi antigens (111).

Inhibition of hemagglutination occurs when free uncombined polysaccharide is added to the antiserum prior to the addition of the antigen modified erythrocytes. This principle has been employed to detect and titrate bacterial polysaccharide in the cerebrospinal fluid of influenza patients (100) and rickettsial polysaccharide in the urine of scrub typhus patients (150).

Several workers have taken advantage of the ability of the erythrocyte to adsorb more than one antigen. These polyvalent antigens may be used as diagnostic aids or in antigenic studies (87, 133, 136, 140, 163, 164, 165). Rapid slide techniques have been developed for use with antigens from Vibrio comma (60), Salmonella typhosa (67), Salmonella schottmuelleri (67), Paracolobactrum spp. (67), Proteus spp.

(67), Mycoplasma mycoides (47), Brucella spp. (67), Trichinella spiralis (161) and Ascaris spp. larvae (99). Indirect hemagglutination has been utilized in the study of many different microorganisms and parasites. Table 1 lists many of the microorganisms and parasites that have been studied by indirect hemagglutination.

Table 1. Microorganisms and parasites studied by means of indirect hemagglutination

Organism	Reference
Aerobacter aerogenes	126
Brucella spp.	24, 67, 89
Diplococcus pneumoniae	9, 100
Escherichia coli	67,79,88,103,104,106, 126,128,129,130,134, 135,138,139,141,142, 143,144,145,146,177, 178
Hemophilus influenzae	100, 101
Hemophilus pertussis	63, 65, 80, 196
Klebsiella spp.	155
Leptospira spp.	33
Mycobacterium bovis	52,62,64,65,81,194
Mycobacterium leprae	112
Mycobacterium paratuberculosis	62, 107
Mycobacterium phlei	62
Mycobacterium tuberculosis	7,10,14,15,16,17,43,44, 45,62,66,70,75,76,78, 120,122,121,124,125, 156,159,160,167,168,169
Mycoplasma mycoides	47
Paracolobactrum spp.	39, 67, 126, 155
Pasteurella multocida	26, 27, 28, 29
Pasteurella pestis	6,37,38,103,105,127,166
Pasteurella tularensis	4, 36, 82, 201
Pfeifferella mallei	12
Pfeifferella whitmori	12
Proteus spp.	67, 100, 126

Table 1 (Continued).

Organism	Reference
Pseudomonas aeuruginosa	71, 103, 126
Salmonella abortus equinum	139
Salmonella anatum	164
Salmonella choleraesuis	155
Salmonella derby	100
Salmonella enteritidis	164
Salmonella montevideo	164
Salmonella newport	164
Salmonella pullorum	100, 164
Salmonella schottmuelleri	67
Salmonella senftenberg	164
Salmonella typhimurium	100, 162, 164, 165
Salmonella typhosa	39,46,67,100,170,171, 173,174
Salmonella spp.	69,189
Serratia marcesans	103
Shigella dysenteriae	131
Shigella flexneri	40, 41
Shigella sonnei	134, 136
Staphylococcus aureus	100, 132, 157
Streptococcus spp.	51,86,100,102,157
streptococcus, group A	51, 87, 157
streptococcus, group C	157
Streptococcus viridans	157
Vibrio comma	60
Vibrio fetus	158,190,191,192
Rickettsia akari	34
Rickettsia conori	34
Rickettsia mooseri	34
Rickettsia pijperi	34
Rickettsia prowzekii	32, 34, 35, 85
Rickettsia rickettsii	34
Rickettsia tsutsugamushi	150
Miyagawanella psittacii	8
Adenovirus	68
Herpes simplex virus	61, 162
Infectious canine hepatitis virus	22
Mumps virus	19
Newcastle disease virus	20
Poliomyelitis virus	115

Table 1 (Continued).

Organism	Reference
Actinomycetes spp.	204
Candida albicans	195
Histoplasma capsulatum	149
Ascaris lumbricoides	97, 99
Ascaris lumbricoides var. suum	97
Clonorchis sinensis	152
Echinococcus granulosus	72, 73
Schistosoma mansoni	96
Toxoplasma gondii	92, 93
Trichinella spiralis	98, 161

METHODS OF PROCEDURE

Source of *Mycoplasma spp.* Isolates

Isolates of *Mycoplasma hyorhinis* utilized were from swine nasal cavities, pneumonic lungs, pericardial fluid, tonsils and arthritic joints. These were isolated from specimens submitted by the Iowa Veterinary Medical Diagnostic Laboratory and by Iowa veterinary practitioners.*

All *Mycoplasma hyorhinis* isolates used in this work have been assigned a number and an abbreviation to indicate the origin of each isolate. Abbreviations used were "Tb" for turbinate, "Lu" for lung, "Tn" for tonsil, "Pc" for pericardial and "Jt" for synovial fluid. The isolates studied in this work were Tb-1, Tb-2, Tb-3, Pc-4, Tn-5, Lu-6, Lu-7, Lu-8, Lu-9, Lu-10, Lu-11, Lu-12, Jt-13, Jt-14, Jt-15, Jt-16, Jt-17, Jt-18, Jt-19, Jt-20 and Jt-21.

Mycoplasma gallinarum isolates were from turkey sinus exudate and pipped turkey embryos.** Two additional *Mycoplasma spp.* isolates used in this work were from a bovine nasal cavity and a pneumonic feline lung.***

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*Appreciation is expressed to Drs. W. P. Switzer and C. L'Ecuyer who recovered some of the isolates.

**Appreciation is expressed to Dr. M. S. Hofstad who recovered these isolates.

***Appreciation is expressed to Dr. W. P. Switzer who recovered these isolates.

Isolation and Propagation

Two types of artificial medium were routinely employed for cultivation of Mycoplasma hyorhinis. One was composed of 80 percent beef heart infusion and 20 percent turkey serum (185). Near the completion of this work it was found that incorporation of 0.5 percent swine gastric mucin in the beef heart infusion portion of this medium improved the growth of Mycoplasma hyorhinis. The second medium consisted of 70 percent beef heart infusion, 20 percent turkey serum and 10 percent yeast autolysate.* The yeast autolysate was prepared by adding one volume of dry yeast** to two volumes of distilled water in a large container. This was incubated at 37°C. for 48 hours. The resulting autolysate was clarified by centrifugation at 2000 r.p.m. for 20 to 30 minutes and stored at -20°C. for up to 4 months. These media were sterilized by filtration through a Selas number 02 or 03 filter, tubed in 6 to 8 ml. amounts in 16 mm. x 125 mm. screw cap pyrex tubes, incubated at 37°C. for 24 hours to check sterility and stored at 4°C. Medium prepared in this manner was found to be satisfactory for use after storage for at least 3 months.

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*Switzer, W. P., Ames, Iowa. Studies on atrophic rhinitis. Private communication. 1959.

**Red Star Yeast and Products Co., Milwaukee, Wisconsin.

Tissue specimens were ground or minced and suspended in tryptose phosphate broth or Dulbeccos phosphate buffer (119). In the case of pericardial, pleural, peritoneal or synovial fluid, the undiluted material was used. In general, 0.2 ml. of a 10 percent tissue suspension or undiluted fluid was inoculated into each tube. Nasal secretions were collected and inoculated with sterile cotton tipped applicators.

Cultures were transferred three times at 48 hour and at 7 day intervals. One-half ml. amounts of culture were used as inocula. One thousand units of penicillin per ml. and 1:4000 thallous acetate were added as bacterial inhibitors for the first three passages (185).

Growth of Mycoplasma hyorhinis produced a slight turbidity and fine sediment in the culture. Verification of the presence of Mycoplasma hyorhinis was made by examination of sediment stained with Giemsa's stain (185) or Stevenel blue stain*.

Some isolates of Mycoplasma hyorhinis were recovered in primary swine kidney cell cultures (179). Fluids from cell cultures exhibiting characteristic cytopathic effect were inoculated into beef heart infusion-turkey serum medium for

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*Daffalla, E. N., Khartoum, Sudan. Stevenel blue stain. Private communication. 1959. (Stevenel blue stain is prepared by mixing 1 Gm. of methylene blue in 500 ml. of distilled water, 500 mg. of potassium permanganate in 500 ml. of distilled water, boiling the combined solutions and filtering with filter paper.)

further study.

Colonies of Mycoplasma hyorhinis were grown on beef heart infusion-turkey serum medium containing 1.5 percent agar (185). In addition 0.42 percent agar was incorporated in beef heart infusion-turkey serum medium plus mucin for growth studies in semi-solid medium (53). This medium was tubed in 6 to 7 ml. amounts in screw top 16 mm. x 125 mm. tubes and inoculated with a straight platinum wire using a slicing motion.

Electron transfer studies were conducted with beef heart infusion-turkey serum medium plus mucin with 0.005 percent 2,3,5-triphenyl-2H-tetrazolium chloride (185) or 1:60,000 methylene blue (54).

Cell suspensions of Mycoplasma hyorhinis for immunization and serological tests were prepared from cultures grown in cotton and gauze stoppered 500 ml. Erlenmeyer flasks containing approximately 200 ml. of beef heart infusion-turkey serum medium plus 1:4000 thallous acetate. These flask cultures were incubated at 37°C. for 36 to 48 hours. Growth was increased up to eight fold by agitation of the flask with a wrist action shaker*.

Figure 1 demonstrates the wrist action shaker and water bath employed. Figure 2 demonstrates the growth of

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*Wrist action shaker, Model BB, Burrell Corp., Pittsburg, Pa.

Figure 1. Wrist action shaker and 37°C. water bath employed to grow Mycoplasma hyorhinis

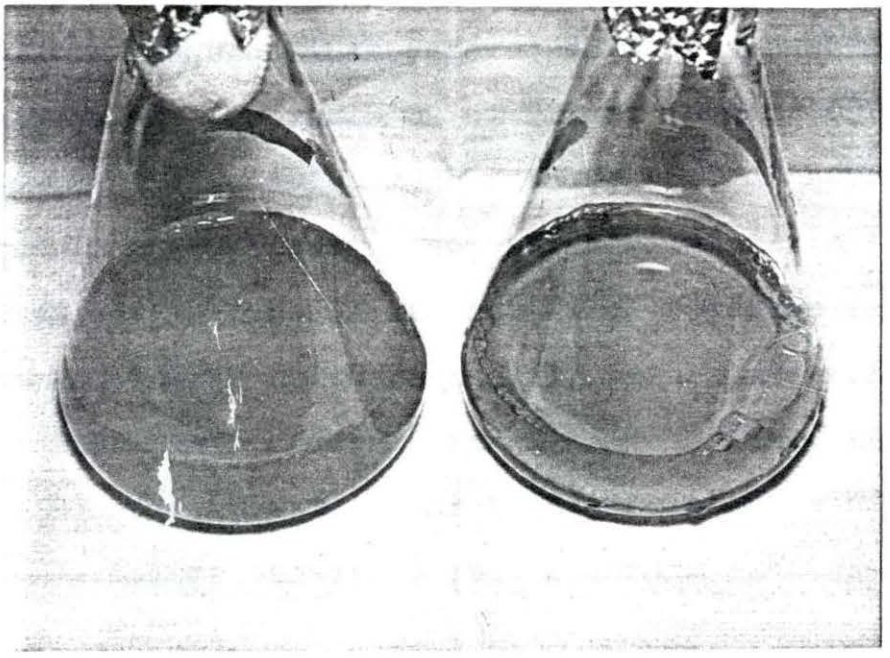

Figure 2. Uninoculated beef heart infusion-turkey serum medium and turbid 36 hour flask culture of Mycoplasma hyorhinis

Mycoplasma hyorhinis in flask cultures. Agitation with a magnetic stirrer increased growth but heat given off by the stirrer raised the temperature in the flask to an undesirable level.

Mycoplasma hyorhinis cells were harvested by centrifugation of the cultures at 16,500 r.p.m. for 30 minutes in a refrigerated angle-head centrifuge. The resulting sediment was suspended in 0.85 percent NaCl solution and centrifuged at 16,500 r.p.m. for 15 to 20 minutes. The sediment was suspended in 0.85 percent NaCl solution and homogenized by gentle mixing with a Tenbroeck tissue grinder. The turbidity was adjusted to the number 10 tube of the McFarland nephelometer scale. This suspension was stored at -20°C. and was usable for at least 6 months. All cell suspensions used in serological tests and antiserum production were prepared in this manner unless otherwise stated.

Antiserum Production

Animals employed for antiserum production were normal appearing White Leghorn roosters, disease free pigs and normal appearing white New Zealand rabbits. The roosters and pigs were raised at the Veterinary Medical Research Institute. The rabbits were secured from a local breeder.

Antiserum for Mycoplasma hyorhinis isolate Tb-1 was produced in young roosters by a series of seven 1 ml.

inoculations of the cell suspension administered intravenously every fifth day. Antiserum against isolate Lu-8 was produced in young roosters, 8 week old pigs and young rabbits. Initially the roosters were given 1 ml. of the cell suspension intravenously and 1 ml. of Lu-8 cells plus an adjuvant. This adjuvant contained paraffin oil, Arlacel A and a dried extract of Mycobacterium butyricum (23). Five subsequent intravenous inoculations given at four day intervals consisted of 1 ml., 2 ml., 3 ml., 4 ml. and 5 ml. respectively. The pigs received 2 ml. subcutaneous and the rabbits 1 ml. subcutaneous inoculations of the adjuvant and Lu-8 cell suspension mixture. In addition the rabbits were given two 1 ml. inoculations followed by four 2 ml. inoculations at four day intervals intravenously. The pigs received 1 ml. intraperitoneal, 2 ml. intraperitoneal, 3 ml. intravenous and 3 ml. intravenous inoculations. The pigs received these inoculations at four day intervals.

Antisera against isolates Lu-7, Pc-4, Lu-6 and Tb-2 were produced in young roosters with a series of four increasing intravenous inoculations at four day intervals. These inoculations started with 1 ml. and increased 1 ml. each time. Antisera for isolate Jt-20 and isolate Jt-14 were produced in a similar manner except that the roosters each received two additional 4 ml. inoculations at four day intervals.

Antiserum for isolate Lu-12 was produced in 1 week old pigs by intraperitoneal inoculation of 1 ml. of whole culture. Antiserum against isolate Tb-3 was produced in a 9 week old pig by a series of four 1 ml. intraperitoneal inoculations at two week intervals of fluid from swine kidney cell cultures showing cytopathic effect.

In each case blood was collected four to six days after the last injection. The sera were harvested, heated at 56°C. for 30 minutes and stored at -20°C.

Indirect Hemagglutination with Washed Erythrocytes

Indirect hemagglutination with washed erythrocytes was initially performed according to the procedures outlined by Middlebrook and Dubos (125), Neter et al. (133) and TePunga (190). Changes were instituted as the optimum conditions for each step were determined. These conditions were determined with cell suspensions of Mycoplasma hyorhinis isolate Tb-1 and its homologous antiserum.

Erythrocytes were obtained by aseptically collecting 1 volume of blood from normal appearing sheep in 1.2 volumes of Alsever's solution (5). This blood could be stored up to 2 months at 4°C. The erythrocytes were harvested by centrifugation at 1800 r.p.m. for 10 minutes and washed three times in conical 15 ml. centrifuge tubes with 0.85 percent NaCl solution. A centrifugation speed of 1500 r.p.m. for

10 minutes was used. The supernatant fluid was removed by vacuum aspiration. After the final wash the erythrocytes were diluted to a 5 percent concentration in 0.85 percent NaCl solution.

Cell suspensions of Mycoplasma hyorhinis adjusted to the number 10 tube of the McFarland nephelometer scale were heated in boiling water for 10 minutes. The coagulated protein was allowed to settle out and the supernatant fluid was mixed with equal parts of 5 percent washed sheep erythrocytes. Adsorption was conducted for 2 hours at 37°C. in 16 mm. x 150 mm. rubber stoppered tubes in a slowly revolving roller drum*. Figure 3 demonstrates this step.

The modified erythrocytes were then centrifuged at 1200 r.p.m. for 8 minutes and washed three times with 0.85 percent NaCl solution at a speed of 800 r.p.m. for 8 minutes. The washed, modified erythrocytes were suspended at a 5 percent concentration in 0.85 percent NaCl solution.

Serial dilutions of antiserum were prepared in 0.5 ml. amounts of 0.85 percent NaCl solution in 13 mm. x 100 mm. serological tubes using a separate pipette for each dilution. A control tube containing 0.85 percent NaCl solution was included with each titration. Five hundredths ml. of the 5

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*Wyble Engineering Development Corporation, Silver Springs, Md.

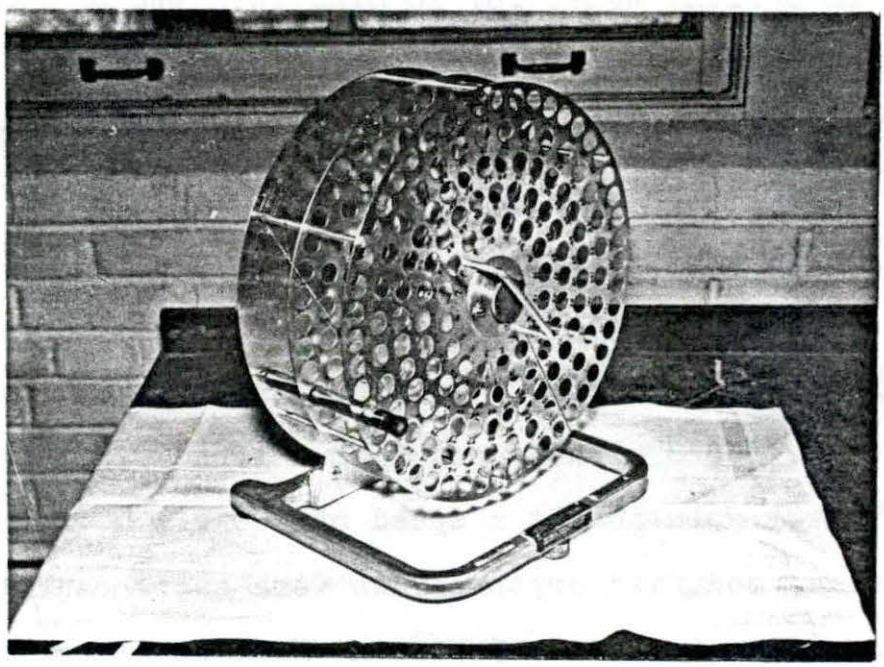

Figure 3. Cell culture roller drum used to agitate washed sheep erythrocytes and Mycoplasma hyorhinis antigen during the adsorption process

percent modified erythrocyte suspension was added to each tube. The tubes were shaken and incubated at room temperature for 2 hours.

Hemagglutination was manifested by the erythrocytes settling out as a shield composed of a smooth coating of erythrocytes on the bottom of the tube. Hemagglutination reactions were graded according to the type of shield formed. Shield formation over the entire bottom of the tube was described as 4+. When the edge of the shield was irregular and covered about three-fourths of the bottom it was considered to be a 3+ reaction. A shield covering about one-half the surface was considered to be a 2+ reaction while reactions with a small ring at the bottom of the tube were designated 1+. Negative reactions such as those obtained with high dilutions of antiserum, normal serum and controls were characterized by a button of erythrocytes in the bottom of the tube. The endpoint of a given titration was selected as the highest dilution in which a 2+ reaction occurred. Figure 4 demonstrates a typical indirect hemagglutination test conducted with Mycoplasma hyorhinis.

Indirect Hemagglutination with Tannic Acid Treated Erythrocytes

Erythrocytes were obtained by collecting 1 volume of blood in 1.2 volumes of Alsever's solution. Erythrocytes aseptically collected in this manner from normal sheep and

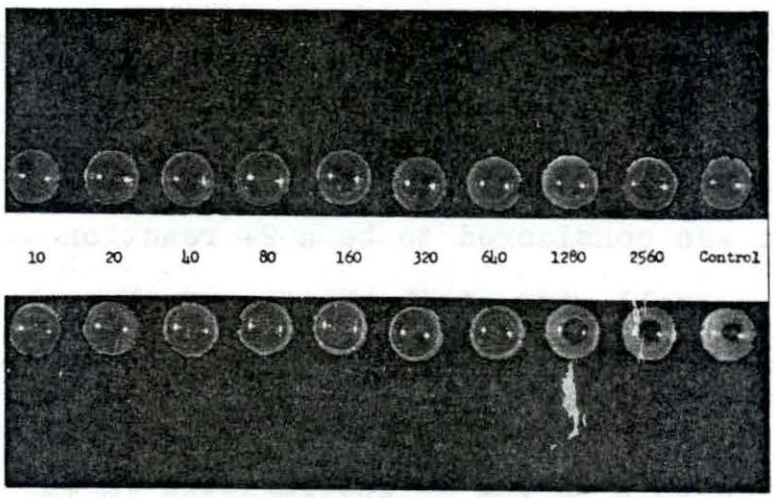

Figure 4. The top row of tubes demonstrates the antiserum dilutions and freshly added Mycoplasma hyorhinis modified erythrocytes. Agglutination of the modified erythrocytes by antiserum dilutions through 1:1280 is demonstrated in the bottom row

swine could be used in this test after storage at 4°C. for up to 3 weeks. Modified erythrocytes were prepared according to the technique described by Stravitsky (176, p. 361).

> The blood cells were washed 3 times with saline. If the supernatant was not clear after these washings the cells were discarded because they were found to be too fragile for the later treatments. 1 ml of packed cells was then diluted with about 40 ml of pH 7.2 buffered saline so that 1 ml of this diluted cell suspension plus 5 ml of distilled water gave a reading of 400 with the #54 filter in the Klott-Summerson colorimeter. These cells often could be kept at 5°C for 2-3 days without extensive hemolysis but as a rule were used within an 18-24 hour period.
>
> Tannic acid. Merck or Mallinckrodt reagent grade diluted with saline was used. The 1/100 dilution was kept at 5°C as a stock solution from which the 1/20,000 acid was made daily.
>
> Buffered saline. The pH 7.2 buffered saline was made by mixing 100 ml of saline and 100 ml of a buffer consisting of 23.9 ml of 0.15 M KH_2PO_4 and 76.0 ml of 0.15 M Na_2HPO_4. The pH 6.4 saline was prepared by mixing 100 ml of saline and 100 ml of a buffer composed of 32.2 ml of 0.15 M Na_2HPO_4 and 67.7 ml of 0.15 M KH_2PO_4. The pH was checked on a pH meter and adjusted as necessary with 0.15 M Na_2HPO_4 or KH_2PO_4.
>
> Preparation of tannic acid cells. 1 ml of cells diluted as above plus 1 ml 1/20,000 dilution of tannic acid were incubated in a water bath at 37°C for 10 minutes. The cells were then centrifuged gently and washed with 1 ml of pH 7.2 buffered saline and resuspended in 1 ml of saline. These tannic acid cells were kept at 5°C for not more than 18 hours before use.
>
> Sensitization of tannic acid-cells. 4 ml pH 6.4 buffered saline plus 1 ml of protein in saline plus 1 ml tannic acid-cells were mixed in this order and kept at room temperature for 10 minutes. The cells were then centrifuged, washed once with 2 ml 1/100 normal rabbit serum and resuspended in 1 ml of 1/100 normal rabbit serum.
>
> Control tannic acid saline cells were prepared by substituting saline for the protein solution in the above mixture. . .

The normal rabbit serum and . . . were diluted with saline.

Doubling dilutions of serum were prepared in 0.85 percent NaCl solution containing 1 percent normal rabbit or swine serum which had been heated at 56°C. for 30 minutes. A separate serological pipette was used for each dilution. The colorimetrically standardized suspension of modified erythrocytes was added at the rate of 0.5 ml. to 0.5 ml. of each serum dilution. The tubes were vigorously shaken and incubated at room temperature until the erythrocytes in control tubes had formed a compact button on the bottom of the tubes.

Hemagglutination was manifested by the erythrocytes settling out in a shield on the bottom of the tube. These shields were composed of a smooth coating of erythrocytes over the entire bottom of the tube. The highest dilution of antiserum producing a definite shield was selected as the titer of that antiserum.

Direct Hemagglutination

Cell suspensions of Mycoplasma hyorhinis diluted to the number 3 tube of the McFarland nephelometer scale with 0.85 percent NaCl solution were used for hemagglutination attempts. Serial dilutions of antigen were made in phosphate

buffered saline*.

Erythrocytes for hemagglutination tests were obtained from blood collected in 2 percent sodium citrate solution. These erythrocytes were centrifuged at 1500 r.p.m. for 10 minutes, suspended in 10 volumes of 0.85 percent NaCl solution and centrifuged at 1200 r.p.m. for 10 minutes. This washing process was repeated with 0.85 percent NaCl solution and then with Alsever's solution. The erythrocytes could be stored up to 3 weeks as a 10 percent suspension in Alsever's solution at $4^{\circ}C$.

Washed erythrocytes were diluted to a 0.75 percent concentration with 0.85 percent NaCl solution and added to 0.5 ml. amounts of Mycoplasma hyorhinis cell suspension dilutions at the rate of 0.5 ml. per dilution. The tubes were vigorously shaken and incubated at room temperature for 2 hours. Hemagglutination was detected by shield formation on the bottom of the tube as opposed to the compact button that occurred in control tubes. Shields were composed of a smooth coating of erythrocytes over the entire bottom of the tube. The highest dilution of Mycoplasma hyorhinis cells producing a definite shield formation was selected as the

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*Cox, H. R., Pearl River, New York. Phosphate buffer. Private communication. ca. 1950. (The buffer was composed of 9.2 Gm. of $NaH_2PO_4 \cdot H_2O$ and 34.6 Gm. of $Na_2HPO_4 \cdot H_2O$ in 2000 ml. of distilled water. The buffered saline contained 8.1 Gm. of NaCl, 50 ml. of buffer and 950 ml. of distilled water.)

hemagglutination titer.

Plate Agglutination

Cell suspensions for use in plate agglutination tests were adjusted to the number 3 tube of the McFarland nephelometer scale by the addition of 0.85 percent NaCl solution. They were then gently homogenized with a Tenbroeck tissue grinder. Two-fold dilutions of antiserum from 1:2 to 1:16 were made in 0.85 percent NaCl solution. Single drops of these dilutions were placed on the glass plate of a standard testing box* with a 0.2 ml. pipette. A 3 mm. platinum loop was used to mix a drop of antigen with each antiserum dilution. The testing plate was rocked gently with a circular motion for 2 minutes at room temperature.

Growth Inhibition by Antiserum

Growth inhibition tests were performed according to the techniques employed with avian (203) and human (56) Mycoplasma. One-half ml. of steril antiserum or sterile normal heated rooster serum and 0.5 ml. of a 48 hour fluid culture were added to 6 ml. of fluid medium. Inhibition of growth was detected after 48 hours incubation at 37°C. by reduced turbidity and sediment in tubes containing antiserum. Differences in pH, reflecting decreased metabolic activity were

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*Jensen-Salsbery Laboratories, Inc. Kansas City, Mo.

detected by the addition of a few drops of phenol red solution to the cultures after incubation.

Growth inhibition tests were also conducted on solid beef heart infusion-turkey serum medium in 15 mm. x 60 mm. petri plates. Blocks of agar from 72 hour cultures were used to inoculate the plates. Small pieces of agar with abundant growth were inverted and streaked across the surface of the fresh medium. Small, sterile filter discs about 8 mm. in diameter were placed on the inoculated surface and saturated with antiserum. Inhibition of growth in a zone around the discs was determined after 72 hours of incubation at 37°C.

Neutralization of Cytopathic Effect in Cell Culture

Several Mycoplasma hyorhinis isolates were inoculated into primary swine kidney cell cultures to compare their cytopathic activity. The swine kidney cell cultures were prepared as previously described (179). One tenth ml. of a 48 hour fluid culture was inoculated into each of four confluent 5 to 6 day old cell sheets. The degree of cell destruction at 2, 3 and 4 days postinoculation was ascertained by inspection of the tubes at X100 by brightfield illumination.

Mycoplasma hyorhinis isolate Tb-1 produced a severe cytopathic effect, therefore it was selected for use in

neutralization trials. Ten-fold serial dilutions of a 48 hour beef heart infusion-turkey serum culture of isolate Tb-1 were prepared in Dulbecco's phosphate buffer. Equal amounts of the various dilutions and antiserum diluted 1:20 were mixed and incubated at 37°C. for 1 hour. One-tenth ml. of each culture dilution and 0.2 ml. of each culture dilution plus antiserum was inoculated into each of four confluent 5 to 6 day old cell sheets. Normal serum was utilized in a control series.

Agar Diffusion Precipitation

Agar diffusion tests were conducted according to the procedure described by Kagan and Bargai (98). Capillary tubes 4 cm. long were prepared from 3 mm. glass tubing, coated with 0.1 percent agar and sealed at one end. Cell suspensions of isolate Tb-3 were heated in a boiling water bath 15 minutes, frozen ten times or disrupted with small glass beads in an Omnimixer* at 14,000 r.p.m. for 5 minutes. One hundredth ml. of each preparation was placed in the bottom of a capillary tube with a blunt 27 gauge needle. This was followed by 0.01 ml. of 0.6 percent agar and 0.01 ml. of antiserum. These were incubated at room temperature in a vertical position. The agar zone between the antigen and

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*Ivan Sorval, Inc., Norwalk, Conn.

antiserum was observed for rings of precipitation for 5 days.

RESULTS

Indirect Hemagglutination with Washed Erythrocytes

Preliminary trials indicated that a heated suspension of Mycoplasma hyorhinis isolate Tb-1 contained antigens which modified washed sheep erythrocytes so that homologous rooster antiserum diluted 1:1024 agglutinated them. Erythrocytes modified with a similar preparation of Mycoplasma hyorhinis isolate Tb-3 were not agglutinated by homologous antiserum. An unheated Mycoplasma gallinarum cell suspension modified erythrocytes so that they were agglutinated by homologous antiserum diluted 1:32. Results of this trial are tabulated in Table 2.

Cell suspensions of isolate Tb-1 and five additional isolates of Mycoplasma hyorhinis were heated for 1 hour in a boiling water bath and the supernatants adsorbed to washed sheep erythrocytes. When these modified erythrocytes were tested against TB-1 and Lu-9 antisera the titers ranged from 0 to 1:80. A 1:80 dilution of Tb-1 antiserum agglutinated isolate Lu-9 modified erythrocytes while a 1:80 dilution of Lu-9 antiserum agglutinated isolate Tb-1 modified erythrocytes. Isolate Tb-3 antiserum diluted 1:10 agglutinated erythrocytes modified with isolate Tb-1 antigen while Lu-12 antiserum, normal heated swine serum or normal heated rooster serum failed to agglutinate erythrocytes modified with

Table 2. Indirect hemagglutination of washed erythrocytes modified with cell suspensions of three mycoplasma isolates

Cell preparations		Homologous antiserum dilutions 8	16	32	64	128	256	512	1024	2048	Control
Tb-1	I[a]	+	+	+	+	+	-	-	-	-	-
	II[b]	+	+	+	+	+	-	-	-	-	-
	III[c]	+	+	+	+	+	+	+	+	±	-
	IV[d]	+	+	+	+	+	-	-	-	-	-
Tb-3	I	-	-	-	-	-	-	-	-	-	-
	II	-	-	-	-	-	-	-	-	-	-
	III	-	-	-	-	-	-	-	-	-	-
	IV	-	-	-	-	-	-	-	-	-	-
M. gal.	I	-	-	-	-	-	-	-	-	-	-
	II	-	-	-	-	-	-	-	-	-	-
	III	+	-	-	-	-	-	-	-	-	-
	IV	+	+	+	-	-	-	-	-	-	-

[a]Sheep erythrocytes modified with supernatant fluids of a cell suspension which had been heated at 121°C. for 2 hours.

[b]Sheep erythrocytes modified with supernatant fluids of a cell suspension which had been heated at 121°C. for 1 hour.

[c]Sheep erythrocytes modified with supernatant fluids of a cell suspension which had been heated in a boiling water bath for 1 hour.

[d]Sheep erythrocytes modified with unheated cell suspension.

this antigen.

Cell suspensions of Mycoplasma hyorhinis isolate Tb-1 were heated for various lengths of time at different temperatures to determine the optimum amount of heat required to liberate the antigens active in this test. Optimum or near optimum activity was obtained by heating for 15 minutes at 70°C., 80°C., 90°C. or heating in a boiling water bath for 5, 10, or 15 minutes. Heating in a boiling water bath for 30 or 60 minutes, heating at 60°C. for 15 minutes and heating at 56°C. for 30 minutes resulted in inferior preparations. Heating in a boiling water bath for 10 minutes was selected as the standard heat treatment.

Sheep erythrocytes were slightly superior to swine or turkey erythrocytes when modified with heated Tb-1 cell suspensions, while bovine, equine and chicken erythrocytes were not satisfactory. Table 3 summarizes these findings.

Table 3. Comparison of modified erythrocytes from various species of animals by means of indirect hemagglutination

	Tb-1 antiserum dilutions							
Source[a]	40	80	160	320	640	1280	2560	Control
Sheep	4+	4+	4+	2+	2+	1+	-	-
Swine	4+	4+	4+	4+	1+	1+	-	-
Cow	-	-	-	-	-	-	-	-
Horse	4+	4+	4+	2+	±	±	±	±
Turkey	4+	4+	4+	2+	1+	-	-	-
Chicken	1+	1+	1+	1+	-	-	-	-

[a]All erythrocytes modified with heated isolate Tb-1 cell suspension.

Attempts were made to preserve sheep erythrocytes with formalin according to the technique described by Tepunga (192) but when these preserved cells were modified with a heated cell suspension of isolate Tb-1 they reacted only with low dilutions of specific antiserum.

The influence of washing of the erythrocytes on adsorption of Tb-1 antigen was determined. Erythrocytes were modified with isolate Tb-1 antigen after having been washed various numbers of times with varying quantities of 0.85 percent NaCl solution prior to adsorption. Unwashed erythrocytes and those washed once did not adsorb a detectible amount of antigen. Erythrocytes washed three times were superior to those washed two, four or five times. The volume of 0.85 percent NaCl solution used to wash the erythrocytes did not significantly alter the adsorptive ability of the erythrocytes but tests performed with erythrocytes which had been washed with approximately ten volumes of 0.85 percent NaCl solution per wash allowed easiest interpretation of agglutination.

Samples of a mixture of erythrocytes and supernatant of heated cell suspension of isolate Tb-1 were withdrawn at various intervals during adsorption. The degree of modification of each sample was determined by testing against specific antiserum. Adsorption appeared to be complete after 1 hour of incubation since at the 2 hour and the 4 hour

incubation intervals adsorption had not increased appreciably. The slightly higher titer obtained with erythrocytes modified for 4 hours was offset by a slight hemolysis of the erythrocytes. This information is summarized in Table 4.

Table 4. Determination of the optimum adsorption time for *Mycoplasma hyorhinis* antigens to modify erythrocytes[a]

Time	Antiserum dilutions 40	80	160	320	640	1280	2560	Control
10 minutes	1+	1+	1+	-	-	-	-	-
30 minutes	3+	3+	2+	1+	-	-	-	-
1 hour	4+	4+	4+	4+	2+	1+	-	-
2 hours	4+	4+	4+	4+	2+	1+	-	-
4 hours	4+	4+	4+	4+	4+	3+		

[a]This test was performed with isolate Tb-1 modified erythrocytes and Tb-1 antiserum.

Agitation of heated Tb-1 cells and washed sheep erythrocytes by means of rotation in a cell culture roller drum for 2 hours at 37°C. resulted in a modified erythrocytes which were superior to those manually agitated at 15 minute intervals, agitated with a wrist action shaker or not agitated. Modified erythrocytes washed with 0.85 percent NaCl solution two or five times after adsorption worked equally well in this indirect hemagglutination test. Unwashed, modified erythrocytes were unsatisfactory.

Incubation of the indirect hemagglutination test at room temperature resulted in higher titers than comparable tests performed at 37°C. Tests incubated at 4°C. had slightly higher titers but negative and control reactions were difficult to read. Modified erythrocytes prepared and diluted in 0.85 percent NaCl solution were usable for 5 to 7 days when stored at 4°C. Modified erythrocytes suspended in Alsever's solution were as active as those suspended in 0.85 percent NaCl solution.

Erythrocytes modified with isolate Lu-8 antigen were agglutinated by low dilutions only of homologous rooster, rabbit and swine antiserum. However, erythrocytes modified with isolate Tb-1 antigen were agglutinated by a 1:160 dilution of Lu-8 rooster antiserum, a 1:320 dilution of Lu-8 rabbit antiserum and a 1:160 dilution of Lu-8 swine antiserum. In an attempt to circumvent this problem, cell suspensions of isolate Lu-8 were diluted 2X, 1X and 0.5X the number 10 tube of the McFarland nephelometer scale prior to heating. Erythrocytes modified with these different concentrations reacted at the same titer with Lu-8 antiserum.

Dialysis of a heated Tb-1 cell suspension in a cellulose casing* against 0.85 percent NaCl solution at 4°C. for 24 hours did not alter its erythrocyte modifying ability.

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*Visking Co., Chicago, Illinois.

Addition of 0.1 ml. of cold 0.1 N trichloracetic acid to 1 ml. amounts of cold cell suspensions of isolate Tb-1 and isolate Lu-8 resulted in precipitates. Erythrocytes modified with dialyzed supernatants of these preparations were agglutinated by only low dilutions of specific antiserum.

Considerable unadsorbed antigen was present in the supernatant fluids after erythrocytes had been modified for 2 hours with Tb-1 antigen. Serial dilutions of this supernatant were made in 1:400 specific antiserum and incubated at room temperature for 30 minutes. This free antigen diluted 1:320 inhibited agglutination of erythrocytes modified with isolate Tb-1.

The most convenient method of preservation of the antigen necessary for modification of the erythrocytes was by freezing unheated cell suspensions of Mycoplasma hyorhinis at -20°C. Antigenic potency was not maintained beyond a week when cell suspensions were stored at 4°C. Merthiolate at a 1:10,000 concentration was satisfactory as a preservative for storage of cell suspensions at 4°C. for short periods of time.

Seventeen Mycoplasma hyorhinis isolates were compared by indirect hemagglutination utilizing a procedure based on the optimum conditions established for indirect hemagglutination with isolate Tb-1. Each isolate was tested against nine different antisera. The results of these comparisons

are tabulated in Table 5.

Indirect hemagglutination tests were performed with sera from pigs with naturally occurring infection from which Mycoplasma hyorhinis was isolated, from pigs with lesions typical of Mycoplasma hyorhinis infection but in which the presence of the organism was not established and from pigs from specific pathogen free herds. Mycoplasma hyorhinis isolate Tb-1 was employed as the source of antigen in these tests. All sera were heated for 30 minutes at 56°C.

Eighteen 6 to 8 week old pigs were experimentally infected with Mycoplasma hyorhinis; thirteen of these received single 2 ml. intraperitoneal inoculations of a 48 hour culture of isolate Tb-1, one received a similar intraperitoneal inoculation of isolate Lu-8 and four received 0.5 ml. intra-articular inoculation of isolates Tb-1, Lu-8, Jt-14 and Jt-20. Sera harvested from these pigs 7 to 21 days post-inoculation were negative when tested against isolate Tb-1 modified erythrocytes.

Mycoplasma hyorhinis was isolated from arthritic joints of seven pigs submitted by Iowa veterinary practitioners. Serum from five of these pigs did not react with isolate Tb-1 modified erythrocytes. However, 2+ reactions were obtained with one sample diluted 1:10 and another diluted 1:20. Sera collected from 13 pigs exhibiting visceral lesions typical of Mycoplasma hyorhinis infection were negative when

Table 5. Comparison of 17 isolates of Mycoplasma hyorhinis by indirect hemagglutination

Antigen	Antisera									
	Tb-1	Tb-2	Pc-4	Lu-6	Lu-7	Lu-8	Lu-9	Jt-13	Jt-14	Control
Tb-1	640[a]	640	40	160	160	160	320	320	160	0
Tb-2	0	80	10	10	40	0	40	40	20	0
Pc-4	40	320	80	320	640	320	40	160	40	0
Tn-5	0	10	10	20	40	10	20	80	10	0
Lu-6	640	640	80	320	320	80	40	640	160	0
Lu-7	0	320	80	160	640	0	40	80	80	0
Lu-8	40	10	40	20	40	20	40	40	20	0
Lu-9	160	20	10	20	20	40	80	40	10	0
Lu-10	160	20	10	20	80	40	20	40	20	0
Lu-11	0	10	10	20	20	10	20	40	10	0
Jt-13	0	10	0	10	40	0	20	40	10	0
Jt-14	0	10	0	10	40	0	20	80	10	0
Jt-15	0	10	10	20	40	10	40	40	10	0
Jt-16	10	10	10	20	20	10	20	40	10	0
Jt-17	0	10	10	20	20	10	20	80	10	0
Jt-18	0	10	10	20	20	10	20	40	10	0
Jt-19	0	10	10	20	20	10	20	40	10	0

[a]Titer as determined by highest serum dilution with a 2+ reaction or higher.

tested against isolate Tb-1 modified erythrocytes. Sixty-eight serum samples collected from pigs in specific pathogen free herds were also negative.

Indirect Hemagglutination with Tannic Acid Treated Erythrocytes

Washed sheep and swine erythrocytes treated with 1:80,000 tannic acid and modified with a cell suspension of Mycoplasma hyorhinis isolate Tb-3 were agglutinated by homologous antiserum. The titers obtained with isolate Tb-3 varied from test to test. The negative and control reactions were difficult to read because the erythrocytes frequently failed to settle satisfactorily.

Attempts were made to liberate an antigen from isolate Tb-3 cell suspensions which would modify the tannic acid treated erythrocytes. Erythrocytes modified with Tb-3 cell suspensions which had been boiled for 15 minutes, frozen 10 times or disrupted with an Omnimixer were not agglutinated by homologous antiserum.

Direct Hemagglutination

Mycoplasma hyorhinis isolate Tb-1 hemagglutinated swine, rat and mouse erythrocytes in a preliminary trial. No hemagglutinating activity could be detected for calf, horse, sheep, dog, cat, human O positive, rooster, turkey, newborn chick, guinea pig or raccoon erythrocytes.

A cell suspension of isolate Tb-1 diluted 1:8 in saline buffered at pH 7.0 and pH 6.5 hemagglutinated swine erythrocytes. Mouse erythrocytes were hemagglutinated by a 1:4 dilution of isolate Tb-1 buffered at pH 6.5 and pH 7.0. Rat erythrocytes were hemagglutinated by isolate Tb-1 diluted 1:16 in pH 6.5 buffered saline. Isolate Tb-1 was less active against these erythrocytes at pH 7.5 and inactive at pH 8.0 and pH 8.5.

The swine erythrocyte hemagglutinating activity of Mycoplasma hyorhinis was studied intensively. Optimum hemagglutination was obtained when tests were performed with 0.75 percent suspensions of swine erythrocytes in 0.85 percent NaCl solution buffered at pH 7 and containing 4 percent normal heated swine serum. The optimum age of culture for use in hemagglutination was determined with cell suspensions prepared with isolate Tb-1 grown in flask cultures for 27, 30, 33, 36, 39, 42, 45, 54, 60, 72, and 96 hours. Erythrocytes from an 8 week old pig were hemagglutinated by a 1:40 dilution of cell suspensions of 36 or 45 hour cultures. Cell suspensions from cultures incubated for shorter or longer periods of time were less active. Erythrocytes from three other 8 week old pigs were not hemagglutinated by 1:5 dilutions of these Tb-1 cell suspensions. Supernatant fluids from cell suspensions of isolate Tb-1 which had been centrifuged at 2000 r.p.m. for 10 minutes or frozen and

thawed six times and centrifuged at 2000 r.p.m. for 10 minutes did not hemagglutinate swine erythrocytes. Washed sheep erythrocytes were hemagglutinated by a 1:64 dilution of a concentrated cell suspension of Mycoplasma hyorhinis isolate Lu-8.

Plate Agglutination

Cell suspensions of Mycoplasma hyorhinis isolate Tb-1 autoagglutinated in normal serum and 0.85 percent NaCl solution. Methods of overcoming this autoagglutination problem were not investigated.

Growth Inhibition by Antiserum

Addition of isolate Tb-1 antiserum to fluid medium resulted in partial inhibition of growth of isolates Lu-10, Tb-2, Lu-9, Pc-4 and Tb-1. Isolate Jt-21 was slightly inhibited by Tb-1 antiserum. No inhibition of colony growth of isolates Lu-10, Tb-2, Lu-9, Pc-4 and Tb-1 was produced by Tb-1 antiserum.

Neutralization of Cytopathic Effect in Cell Culture

Twenty-two isolates of Mycoplasma hyorhinis, one feline Mycoplasma sp. isolate, one bovine Mycoplasma sp. isolate and two Mycoplasma gallinarum isolates were inoculated into primary swine kidney cell cultures. Nine of 12 Mycoplasma

hyorhinis isolates of lung origin produced a cytopathic effect. Eleven of these were recovered from the cell culture fluids in beef heart infusion-turkey serum medium. Three turbinate Mycoplasma hyorhinis isolates produced a cytopathic effect and were recovered from the cell culture fluids. Three of five synovial fluid isolates produced cytopathic effect. All five isolates were recovered from the cell culture fluids. An isolate of Mycoplasma hyorhinis of tonsil origin failed to produce a cytopathic effect while a pericarditis isolate produced a cytopathic effect. Both isolates were recovered from the cell culture fluids.

The feline lung and bovine nasal isolates did not produce a cytopathic effect even though the bovine isolate was recovered from the cell culture fluids. The two isolates of Mycoplasma gallinarum produced a cytopathic effect similar to that produced by Mycoplasma hyorhinis and were recovered from the cell culture fluids.

One-tenth ml. amounts of a forty-eight hour culture of isolate Tb-1 produced cytopathic effects in swine kidney cells through dilutions of 10^{-3}. Antiserum against isolate Tb-1 inhibited the development of this cytopathic effect for about 12 hours.

Agar Diffusion Precipitation

Agar diffusion studies were performed with cell suspensions of isolate Tb-3 which had been boiled, frozen and mechanically disrupted. Negative results were obtained when these preparations and untreated cell suspensions of Tb-3 were tested against Tb-3, Tb-1 and Lu-12 antisera. Tests performed with isolate Tb-3 cell suspensions and serial dilutions of Tb-3 and Tb-1 antiserum from undilute to 1:40 were negative. Negative results were obtained with normal rooster and normal swine sera.

Growth, Metabolic and Morphological Studies

Growth of most turbinate, lung and pericardial isolates of Mycoplasma hyorhinis in fluid medium is manifested by a slight turbidity and a fine deposit of sediment. Turbidity in cultures of these isolates could frequently be detected 15 to 20 hours after inoculation as a faint 6 to 10 mm. band about 8 to 10 mm. below the surface of the medium. Growth appeared to spread throughout the medium for the first 36 to 48 hours postinoculation. Little increase in growth occurred beyond this time. Isolates varied to a considerable degree in the amount of turbidity and sediment produced.

Mycoplasma hyorhinis isolates recovered from the synovial fluid of arthritic joints usually grew more slowly. Turbidity developed throughout the medium in young cultures

of these isolates with maximum growth occurring in 5 to 7 days. Sediment in these cultures occurred as a granular deposit covering the bottom of the culture tube. Growth of these isolates was increased two to four times by the addition of swine gastric mucin to the beef heart infusion-turkey serum medium.

Giemsa stained preparations of the turbinate, lung and pericardial isolates resembled those described in previous publications (181, 185). Similar preparations of the synovial fluid isolates were characterized by the presence of numerous spherical, bluish staining bodies varying from 5 to 15 microns in diameter. These bodies appeared to be identical to those previously observed in this laboratory.* Typical Mycoplasma hyorhinis cells were scattered throughout these preparations and were clustered around the large bodies.

Turbinate, lung, pericardial and synovial fluid isolates developed typical colonies in 48 to 72 hours on solid beef heart infusion-turkey serum medium. Agitation of flask cultures of most turbinate, lung and pericardial isolates increased growth up to eight-fold. Flask cultures of synovial fluid isolates were not greatly stimulated by this agitation. Growth occurred in flask cultures of synovial

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*Switzer, W. P., Ames, Iowa. Studies on atrophic rhinitis. Private communication. 1959.

fluid isolates as a granular suspension while most turbinate, lung and pericardial isolates grown in flask cultures were densely turbid.

Stab cultures of isolates Tb-1 and Lu-8 in semisolid medium grew as a fine granular white streak in the top half of the medium. Similar cultures of isolates Jt-14 and Jt-20 were characterized by growth at all depths of the medium.

Isolates Tb-1 and Lu-8 reduced triphenyl tetrazolium whereas isolates Jt-14 and Jt-20 produced only a slight reduction. Growth of isolates Tb-1 and Lu-8 in fluid medium containing methylene blue could be detected as early as 6 hours postinoculation by a faint band of reduction about 4 to 6 mm. wide approximately 8 to 10 mm. below the surface of the medium. After 48 hours incubation at 37°C. all but a thin band of methylene blue at the top of the culture had been reduced. No evidence of methylene blue reduction could be detected at 6 hours in cultures of isolates Jt-14 and Jt-20. However at 48 hours reduction had occurred throughout the lower three-fourths of these cultures.

Two 8-week-old pigs inoculated intraperitoneally with 2 ml. of 48 hour beef heart infusion-turkey serum plus mucin cultures of isolates Tb-1 and Lu-8 developed acute serositis and arthritis. Similar inoculations with isolates Jt-14 and Jt-20 resulted in no significant reactions. Intra-articular inoculations of young pigs with 0.5 ml. amounts of culture

of these same isolates produced an arthritis from which each was recovered.

DISCUSSION

An indirect hemagglutination test has been developed for use in the study of Mycoplasma hyorhinis. At the time this work was initiated there was no published information on the use of this serological technique with any Mycoplasma. However during this study a report on the use of this test with Mycoplasma mycoides was published (47). Determination of the conditions necessary for the test resulted in a reliable serological method for the study of Mycoplasma hyorhinis. This test was successfully employed in the comparison of various isolates of Mycoplasma hyorhinis. The application of this technique for the detection of antibodies in serum from naturally or experimentally infected swine has not been adequately evaluated. The results obtained with hemagglutination, agar diffusion precipitation, plate agglutination, growth inhibition by antiserum and neutralization of cytopathic effect in cell culture were not encouraging.

Conditions necessary for modification of the erythrocytes and preparation of the antigens for use in the indirect hemagglutination test were investigated. Sheep erythrocytes have frequently been employed in indirect hemagglutination tests(130, 60, 65, 125, 201) so they were tried first. However, swine and turkey erythrocytes were later found to be nearly as satisfactory as sheep

erythrocytes. This is the first time that swine or turkey erythrocytes have been employed in indirect hemagglutination. Sheep erythrocytes were found to be usable after storage in Alsever's solution at 4°C. for at least 3 months. Erythrocytes washed less than three times prior to the adsorption process were apparently coated with residual serum components which prevented the adsorption of the Mycoplasma hyorhinis antigens.

Although some antigen was available in unheated cell suspensions of Mycoplasma hyorhinis, heating for 5 to 15 minutes at temperatures ranging from 70°C. to 100°C. liberated the maximum amount of antigen. Additional heating partially destroyed the antigen.

Optimum modification of the erythrocytes was obtained after incubation with the antigen for 1 to 2 hours at 37°C. in a slowly revolving cell culture roller drum. These modified erythrocytes could be stored for only 5 to 7 days at 4°C. Attempts to overcome this short storage life by modifying formalin preserved erythrocytes were unsuccessful.

Since free antigen reacted with antibody and prevented hemagglutination, the modified erythrocytes were routinely washed three times. It was found that antigen-modified erythrocytes could be washed at least 5 times with no apparent loss in reactivity.

Considerable antigenic variation was detected in the 17

isolates of Mycoplasma hyorhinis studied with the indirect hemagglutination test. This variation in antigenic composition was apparently quantitative since in all cases there was some degree of cross reaction. The lung, turbinate and visceral lesion isolates varied as to the relative titer at which they reacted with a given antiserum. This indicates that these particular isolates have two or more antigens which can be adsorbed to erythrocytes.

Certain antisera reacted at higher titers with heterologous than with homologous antigens. This suggests that some isolates were deficient in antigenic substance but that continued antigenic stimulation by these deficient antigens in the hyperimmunization process resulted in near maximum antibody production. Therefore, it is possible that greater antigenic variation occurred in each isolate than was indicated by the respective amount of antibody present in homologous antiserum. The possibilities that the antigenic capabilities of an isolate varied with continued passage in artificial medium or with the age of the culture were not explored.

The poor reactivity of Lu-8 modified erythrocytes was not improved by increasing the concentration of antigen. It was also noted that the erythrocytes modified with an extremely concentrated heated cell suspension exhibited increased fragility after washing. Therefore, increasing the

concentration of the heated cell suspension does not appear to solve the antigen deficiency problem encountered with certain isolates. It is possible that incomplete antigens or nonspecific inhibitors present in small amounts in the Mycoplasma hyorhinis cell combine with receptors on the erythrocytes and prevent adsorption of the antigens. Increased concentration of these factors might counterbalance the increase in antigen obtained from concentrated heated cell suspensions. It was also observed that supernatants of heated cell suspensions of isolate Tb-1 were quite turbid, whereas those of isolate Lu-8 were much less turbid. The turbidity of the supernatant appeared to be an index of the amount of soluble antigen available for adsorption to the erythrocytes.

The results obtained in trials to establish the optimum conditions for this test give some indication of the chemical nature of these Mycoplasma hyorhinis antigens. Most indirect hemagglutination tests conducted with washed sheep erythrocytes involve a soluble polysaccharide antigen (52, 66, 130, 140, 194, 201). In contrast those employing tannic acid treated erythrocytes involve protein antigens (16, 41, 51, 103, 127, 168). The Mycoplasma hyorhinis antigens are heat stable and are not precipitated by trichloracetic acid. Also, as with many other indirect hemagglutination tests involving polysaccharide antigens, at least an hour at 37°C.

is required to adequately modify washed sheep erythrocytes. Thus it appears that the antigen or antigens active in this indirect hemagglutination test are probably polysaccharides. Since structurally related polysaccharide antigens frequently cross react in serological tests (11) the antigens studied with this indirect hemagglutination test may have cross reacted.

All of the synovial fluid isolates as well as isolates Tn-5 and Lu-11 reacted to each of the nine antisera with no more than a one dilution deviation. The rest of the isolates varied considerably in their reactivity. On the basis of this consistent serological relationship it appears that all of the synovial fluid isolates were antigenically similar. Agglutinin adsorption techniques should assist in determining the relationships of the other isolates.

Synovial fluid isolates of Mycoplasma hyorhinis require 5 to 7 days to achieve maximum growth in stationary fluid cultures, their growth is increased two to four times by swine gastric mucin and to a lesser extent by yeast autolysate* and they are non-pathogenic for swine by intraperitoneal inoculation. Giemsa stained preparations of these isolates contain numerous spherical, bluish-purple staining bodies 5 to 15 microns in diameter surrounded by

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typical Mycoplasma hyorhinis cells. These synovial fluid isolates have a low oxygen requirement since agitation of flask cultures does not increase growth and they grow throughout the depth of semisolid medium or in the bottom two-thirds of stationary fluid cultures. These isolates slowly reduce methylene blue and triphenyl tetrazolium. Isolates Tn-5 and Lu-11 were antigenically and morphologically similar to the synovial fluid isolates.

Turbinate, visceral lesion and most lung isolates of Mycoplasma hyorhinis studied required 48 to 72 hours to reach maximum growth in stationary fluid cultures and their growth was only slightly stimulated by the addition of swine gastric mucin. They were stimulated by agitation of flask cultures. Representative isolates of this group grew in the upper one-half of semisolid medium, grew initially in the upper portion of stationary fluid cultures and rapidly reduced triphenyl tetrazolium and methylene blue. A severe serositis and arthritis was produced by intraperitoneal inoculation of typical cultures of this group into susceptible young swine.

Serological, morphological, metabolic and pathogenic differences between Mycoplasma hyorhinis isolates of lung, turbinate and visceral lesion origin and those of synovial fluid origin indicate that there are two distinct types of organisms. Table 6 summarizes the various properties of

Table 6. Comparison of morphological, serological, growth, metabolic and pathogenic properties of two types of Mycoplasma hyorhinis isolates

Characteristic	Type I[a]	Type II[b]
Maximum growth obtained in fluid[c] cultures in 2 to 3 days	+	-
Maximum growth obtained in fluid cultures in 5 to 7 days	-	+
Smooth deposit of sediment in fluid cultures	+	-
Granular deposit of sediment in fluid cultures	-	+
Growth stimulated 2 to 4 times by swine gastric mucin	-	+
Growth stimulated by yeast autolysate	-	+
Minute raised translucent colonies about 0.1 mm. in diameter develop in 2 to 3 days on solid medium	+	+
Up to an eight-fold increase in growth is obtained by agitation of flask cultures	+	-
Growth occurs throughout all depths of stab cultures	-	+
Growth occurs in upper one-half only of stab cultures	+	-
Reduce tetrazolium in less than 48 hours	+	-

[a]Type I includes turbinate, visceral lesion and most lung isolates studied in this work.

[b]Type II includes synovial fluid isolates studied in this work.

[c]Beef heart infusion-turkey serum medium plus mucin.

Table 6 (Continued).

Characteristic	Type I[a]	Type II[b]
Reduce methylene blue in less than 48 hours	+	-
Pathogenic for young pigs when inoculated intraperitoneally	+	-
Giemsa stained preparations contain spherical bluish staining bodies 5 to 15 microns in diameter in addition to the typical bluish purple coccoid rods which measure 0.3 to 0.6 microns in length	-	+
Antigenically homogeneous when tested by indirect hemagglutination	-	+

these two types of Mycoplasma hyorhinis. In previous work several isolates of Mycoplasma hyorhinis similar to the above described synovial fluid isolates were recovered from the nasal cavities and joints of swine. These were recovered in beef heart infusion-turkey serum medium plus 10 percent yeast.* Isolates Tn-5 and Lu-11 also appear to belong to this group.

The lung, turbinate and visceral lesion isolates of Mycoplasma hyorhinis studied in this work appear to be identical to those described in previous work (25, 30, 42, 108,

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109, 151, 181, 182, 183, 184, 185, 186, 199, 200). It is felt that with continued work the synovial fluid isolates and similar isolates may be placed in a separate species. Continued study of Mycoplasma hyorhinis in experimental pigs and in artificial medium should allow determination of this point.

The diagnostic application of the indirect hemagglutination test has not been adequately evaluated. Results obtained with sera from 18 pigs experimentally infected with Mycoplasma hyorhinis were negative. Only two of these pigs developed severe symptoms. It has been observed that pigs over 8 weeks of age are difficult to experimentally infect with this agent (181). Thus age may have played a part in the poor antibody response of these pigs. Serum from two of seven proven field cases of Mycoplasma hyorhinis were positive in low dilutions. Sera from sixty-eight specific pathogen free pigs and 13 pigs with fibrinous serositis were also negative.

Concurrent isolation attempts and serological studies should be carried out with swine naturally and experimentally infected with Mycoplasma hyorhinis. Serological surveys in herds of swine with a history of Mycoplasma hyorhinis infection should also be conducted. In this manner it should be possible to adequately evaluate the

diagnostic potential of the indirect hemagglutination test for Mycoplasma hyorhinis.

Indirect hemagglutination tests conducted with Mycoplasma hyorhinis modified tannic acid treated sheep and swine erythrocytes were not successful because of difficulties encountered in reading the negative and control reactions.

Although Mycoplasma hyorhinis hemagglutinated swine, rat, mouse and sheep erythrocytes, this activity was low and rather erratic. For this reason it does not appear that a direct hemagglutination inhibition test will be practical.

Preliminary trials with plate agglutination were inconclusive since the cell suspensions autoagglutinated. The results obtained by Cole (42) and Ose (151) suggest that continued effort with this test might prove successful.

Inhibition of growth in fluid medium by antibody corroborated the findings of Switzer* and Ose (151). The retardation of the cytopathic effects in cell cultures was probably a reflection of the inhibition of growth in the cell culture fluids. It is significant that in neither test was the inhibition of growth complete. This is in contrast to the generalization made that neutralization of Mycoplasma

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by antiserum indicates a relationship to viruses (55).

Swine kidney cell cultures have proven to be a highly effective means of isolating Mycoplasma hyorhinis from swine lungs (110). However, certain isolates of Mycoplasma hyorhinis survive and multiply in swine kidney cell cultures without producing detectible cell damage. This is particularly true of the synovial fluid isolates.

The negative results obtained with the agar diffusion test may have been due to the selection of isolate Tb-3 as the antigen source because negative and erratic results were also obtained with this isolate in indirect hemagglutination tests.

SUMMARY AND CONCLUSIONS

An indirect hemagglutination test was developed for use with Mycoplasma hyorhinis. Washed sheep erythrocytes modified with antigens from heated Mycoplasma hyorhinis cell suspensions were agglutinated by hyperimmune rooster, rabbit or swine antiserum in dilutions from 1:320 to 1:640. Low dilutions of serum from two pigs with natural infections agglutinated Mycoplasma hyorhinis antigen modified erythrocytes. Negative results were obtained with sera from 36 other pigs which had been naturally or experimentally infected.

The indirect hemagglutination test revealed considerable antigenic variation among 17 isolates of Mycoplasma hyorhinis. Nine isolates, seven of which were recovered from synovial fluid, were antigenically similar. This group of nine isolates possessed growth, metabolic, morphological and pathogenic properties different from the other eight isolates. The remaining eight isolates exhibited varying degrees of antigenic relationship to each other.

Other serological techniques which were evaluated included serum neutralization of cytopathic effect in cell culture, plate agglutination, direct hemagglutination, agar-diffusion precipitation and growth inhibition by antiserum. Preliminary trials with these tests were not promising.

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