BARD

FINAL REPORT

PROJECT NO. I-939-85

New Genetic and Serological Approaches in Diagnosis of Mycoplasmosis in Poultry

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1990

Standard BARD Cover Page for Scientific Reports

Date: JAN. 10, 1990

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BARD project No. I-939-85

Title

NEW GENETIC AND SEROLOGICAL APPROACHES IN DIAGNOSIS OF
MYCOPLASMOSIS IN POULTRY

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Project's starting date: AUGUST 7, 1986

Type of Report: 1st Annual 2nd Annual Final x

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C. ABSTRACT

Mycoplasma infection in poultry is of worldwide concern. Control strategy is based on maintenance of turkey and chicken breeder flocks free of infection with pathogenic mycoplasmas. Conventional methods of monitoring flocks for Mycoplasma gallisepticum and M. synoviae are based on serological tests which are subject to a high degree of non-specificity, with confirmation of suspicious reactors by culture or supplemental serological methods. This project made use of recent advances in basic molecular biology research to improve test procedures. Two approaches were used: 1) improvement of specificity of reagents for serodiagnosis; 2) direct detection of mycoplasmas in clinical specimens by specific gene probes.

Examination of genetic relatedness and variation among species and strains constituted the first phase in our research. Marked heterogeneity was detected in the M. gallisepticum species when tested by restriction enzyme patterns. Using the method of genomic fingerprinting with highly conserved genes, strains were found to fall into intraspecies clusters. These methods were found to be effective in identification of the M. gallisepticum F vaccine strain and in other epidemiological studies. In addition we could demonstrate shared genomic sequences in M. gallisepticum and M. synoviae. Some of these shared sequences may encode for common epitopes and thus account for cross-reactions between the species.

Our studies on serodiagnosis and immunogenicity reaffirmed the difficulties due to non-specific reactions. Immunoblot studies allowed the identification of species-specific polypeptides which were used in a DOT-ELISA test to detect infection. Evaluation of the purified test antigens on clinical samples showed the antigens to effectively detect natural infection with no cross-reactions. Improved rapid slide agglutination antigens were also prepared, using mycoplasma organisms propagated in serum-free media.

Specific DNA probes for M. gallisepticum (pMG4) and M. synoviae (pSN27) were selected from genomic libraries prepared in the pUC13 vector. Studies with probe pMG4 indicated the ability of the probe to detect M. gallisepticum in tracheal samples from experimentally or naturally infected chickens and turkeys. Dot-blot hybridization with the probe detected the pathogen at an early stage of infection when no other specific indications were available, and under conditions in which isolation of the organism was not technically possible. The gene probes were further used for rapid identification of mycoplasmas in enrichment cultures.

D. OBJECTIVES AS SET FORTH IN BARD PROPOSAL

1. Serological diagnostic methods

- A. Improvement of existing agglutination antigens
- B. Identification of specific and non-specific components of reaction between mycoplasma antigens and chicken sera.
- C. Improved antigen for ELISA system.

2. Genetic diagnostic methods

- A. Cloned rRNA gene probes
- B. Non-radioactive methods for probe detection
- C. Development of specific gene probes
- D. Application of gene probes

3. Field testing of improved antigens, serological tests and gene probes.

E. Scientific Report

INTRODUCTION

Rapid and reliable diagnosis of M. gallisepticum and M. synoviae is the keystone of mycoplasma control programs in chicken and turkey breeding flocks, and an essential element in the modern intensive poultry industry. This project relates to the improvement of current serological diagnostic methods and to testing the feasibility of the innovative gene probe concept for detection of mycoplasma DNA. In the systematic approach to this subject, the genetic and serological variation within the M. gallisepticum and M. synoviae species was studied, as well as the relationship between the two species. At each stage of the study application to field diagnosis was evaluated, using clinical specimens available to each of the laboratories.

MATERIALS AND METHODS

Bacterial strains: The origin of strains employed, strain designation and relevant biological properties are described in detail in our publications, in particular, publication #14 (included in its entirety in the Appendix) and publication #17. The source of field isolates from the U.S.A. is described in publication #10, those from Israel in publication #14 and those from Australia in publication #9.

Strains used for probe preparation were M. gallisepticum S-6 (isolated by R. Yamamoto, University of California, U.S.A.) and M. synoviae WVU1853 (type strain, isolated by N. O. Olson, Univ. of West Virginia, U.S.A.). Experimental infection was carried out with M. gallisepticum R (isolated by D. G. Ritchie, Georgia, U.S.A.). The origin of other strains used for testing specificity of the probes has been described elsewhere (publications #7, 14). Mycoplasma species other than the above were obtained from E. A. Freundt (FAO/WHO Collaborating Centre for Animal Mycoplasmas, University of Aarhus, Denmark) and are listed in detail elsewhere (publication #7).

Growth conditions: M. gallisepticum and M. synoviae strains were grown in Frey's medium (Olson, 1984); other avian mycoplasmas were grown in Hayflick's mycoplasma medium (Hayflick, 1965) supplemented with glucose or arginine, according to the nutritional requirements of the organism. M. pneumoniae FH, M. genitalium M30, M. pirum 70-159, Spiroplasma citri Maroc R8A2 and Acholeplasma laidlawii (oral strain) were from our Departments' collections and were grown as described elsewhere (Yogev and Razin, 1986). The organisms were harvested after 24-96 h of incubation at 37°C depending on the strain) and the pellets were washed once in 0.25M NaCl containing .01 M NaEDTA, pH 8.0. The washed pellets were stored at -20° C until used for DNA extraction.

Isolation of mycoplasmas for surveillance or determination of status in experimental infection was routinely made by tracheal swab, inoculated into Frey's medium.

Serological testing: For routine screening, serum samples were tested for the presence of antibodies to M. gallisepticum or M. synoviae by the rapid slide agglutination (RSA) test using commercial stained antigen, following the manufacturer's instructions. Laboratory-prepared RSA antigens were used

according to the protocols described in the relevant publications. Positive samples in the RSA test were tested for activity in the hemagglutination inhibition (HI) test, using standard methods (U.S.D.A., 1985). The HI antigen was prepared from M. gallisepticum strain A5969, except as noted.

Commercial Elisa kits were obtained from Whittaker M.A. Bioproducts, Walkersville, MD or from Agritech Systems, Inc, Portland, ME, and were used as directed by the manufacturers, with the exceptions noted in Publication #3.

Preparation of Rapid Slide Agglutination test antigen:

1) Serum medium: Different batches of M. gallisepticum RSA antigens were prepared according to standard methods described previously (Glisson et al, 1984). Modifications in media components and growth procedures are described in detail elsewhere (publications #1 and #2).

2) Liposome (LPS) antigen: Three batches of RSA antigen were prepared by using liposomes in regular mycoplasma medium without serum supplement. Three percent by volume of the liposome mixture and 5% by volume of 8% bovine serum albumin (BSA) in 0.025 M HEPES buffer were added to the medium. The effect of various growth parameters on antigen yield and properties was studied, as described in detail in publication #2.

Separation of mycoplasma and liposomes was carried out by sucrose gradient centrifugation. The resuspended cell pellet including the liposomes (approximately 10 ml) was placed on top of 20 ml of a sterile 25% (w/v) sucrose cushion in a 50 ml centrifuge tube. The liposome-free pellet obtained by centrifugation at 20,500 x g for 30 min was resuspended in phenolized phosphate buffer and standardized as described elsewhere (publication #2).

Preparation of liposomes: The liposomes were prepared by a previously reported method (Satoshi et al, 1986), as described in publication #1. Phosphatidylcholine and cholesterol were suspended in water, and sonicated to obtain a milky white suspension which was stabilized by the addition of 0.4M sucrose. This suspension served as the liposomes for the growth medium.

Specificity and Sensitivity Trials for diagnostic antigens:

1) Commercial Elisa kits and RSA antigens: Mycoplasma-free Leghorn pullets were vaccinated at 13 wks of age with M. gallisepticum or with commercial or laboratory-prepared vaccines, as described in publication #3. Birds were bled periodically for 10-12 wks post-vaccination. Serum samples were tested

with two commercial ELISA kits, various commercial or laboratory prepared RSA antigens, and the HI test. Any birds showing a suspected positive serological reaction were cultured for mycoplasmas.

2) Experimental liposome antigens: Specificity of LPS antigens was compared to that of serum-grown RSA antigens in trials carried out in general as described for ELISA kits, as described in detail in publication #1. Sensitivity of several LPS antigens was compared to serum-grown antigens by testing serum samples from M. gallisepticum-infected Leghorn chickens on a daily basis for 3 wks post-infection (PI). Comparison was made to results in the HI test, and infection was confirmed by isolation. Details of experimental design are reported in publication #2.

Data analysis of comparison of diagnostic tests: Statistical analysis of serological results was carried out by chi-square analysis ($p < 0.01$) and by calculation of Youden's J index, as described in publications #1 & 3.

SDS-PAGE gel electrophoresis: Mycoplasma cells were solubilized in 10mM Tris containing 0.2% (w/v) sodium deoxycholate, 1.0% (v/v) Triton X-100, 10 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride (pH 7.8) for 30 min at 37°C. Solubilized mycoplasma proteins were assayed for protein content, mixed with lysis buffer (125 mM Tris, 4% SDS, 0.1% 2-beta-mercaptoethanol and 20% glycerol, pH 6.8, and heated to 100°C for 5 min, as described in publications #2 and #5. Samples (30 ug/well) were applied to 0.75 mm thick, 4% stacking /10% running SDS-PAGE gels and run overnight at 4 milliamps constant current/gel using the buffer system of Laemmli (1970).

Immunoblot analysis: Two procedures were used for immunoblotting. In the U.S.A., proteins were transferred to 0.45 um nitrocellulose (NC) filter (Bio-Rad, Richmond, CA) at 50 volts constant voltage for 2 hrs (Towbin et al, 1979). Total transferred protein was visualized using 0.1% amido black stain. NC strips with transferred mycoplasma proteins were blocked by incubation for 3 hrs at room temperature (RT) in TBS containing 4% fetal calf serum. Primary antisera were diluted 1:80 in TBS containing 1% swine serum and 1% calf serum (TBS-serum). The diluted antisera were allowed to stand 15 min before incubating with NC strips for 3 hrs at RT and then overnight at 4°C. This was followed by four 8-min washes in TBS -Tween.

Affinity purified rabbit anti-chicken IgG (H+L chains)-horseradish peroxidase (Zymed Lab, San Francisco, CA) was diluted 1:1000 in TBS-serum and incubated with NC strips for 4 hrs at RT. This was followed by 4 washes as above and then a 3-min wash in TBS. Immunoblots were developed by incubation for 5 min in a solution containing H_2O_2 and 4-chloro-1-naphthol (BioRad) as directed by the manufacturer.

In Israel, the washed cell pellets were frozen and thawed twice and then suspended in TEP buffer (100mM Tris-HCl, pH 7.4, 10mM EDTA, 1mM phenyl-methylsulfonyl fluoride). The resulting suspension was subjected to electrophoresis on 10% polyacrylamide gels, according to Laemmli (1970). Transfer of proteins from the gel to NC sheets was as described above, using the Bio-Rad Trans-blot Cell apparatus (Bio-Rad Laboratories, Richmond, CA). Unoccupied sites on the sheet were blocked with 20% (v/v) of fetal calf serum (FCS) in TBS (10 mM Tris-HCl, pH 8.0, 150 mM NaCl). The blots were incubated with rabbit antibody to either M. synoviae WVU1853 or to M. gallisepticum PG-31, diluted 1:750 in 20% FCS in TBS. The antisera were obtained from the FAO/WHO Collaborating Centre for Animal Mycoplasmas, Aarhus, Denmark. To remove possible antibodies to growth medium components, the two antisera were preadsorbed by adding 10% (v/v) of complete Frey's medium to antisera diluted 1:750 in 20% FCS in TBS, and incubating at RT with gentle shaking for 4 h. The blots were incubated with the preabsorbed antiserum for 1 h at room temperature with gentle shaking followed by 10 min washings, first with TBS, then with TBS + 0.1% Nonident P-40 (Sigma) and finally with TBS. The blots were then incubated for 30 min at RT with alkaline phosphatase conjugated-goat anti-rabbit IgG(Fc) (Promega Biotec, Madison, WI), diluted 1:7500 in 20% FCS in TBS. Bound antibody was detected by staining with a solution of 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium (Promega).

Purification of proteins from SDS-PAGE: After staining with comassie blue, protein bands of interest were cut from SDS-PAGE gels (10%) with a razor blade and destained completely. Protein was electroeluted from the cut gels using a model 422 electro-eluter (BioRad, Richmond, CA). Eluted protein was stored at -70°C until use.

Dot-ELISA protocol: Purified antigens were brought to a concentration of 1% KOH by the addition of a 25% KOH solution and applied in 2.0-3.0 ul dots on 0.2 um gridded NC (Micro Filtrations Systems, Dublin, CA). After drying at RT the NC was washed for 5 min in Tris buffered saline (TBS; 20mM Tris, 500 mM NaCl, pH 7.5). After overnight incubation of filter in TBS containing 4% fetal calf serum, reaction with primary antisera (diluted 1:50 in TBS containing 1% calf serum/1%swine serum/1% Frey's medium) was carried out for 75 min at RT with gentle rocking. After washing 4 times/5min with TBS containing 0.05% Tween-20 (TBS-Tween), reaction was carried out with anti-chicken IgG conjugate as for the primary antiserum. After 3 washings with TBS-Tween and one wash with TBS, color development was performed by incubation for 5 min in a solution containing H₂O₂ and 4-chloro-1-naphthol (BioRad) as directed by the manufacturer.

DNA preparation and cleavage: DNA was extracted and purified by the method of Marmur (1961). The DNA was digested by restriction enzymes (New England BioLabs, Inc., Beverly, Mass.) for 2 h at 37°C with buffer mixtures recommended by the manufacturer.

Electrophoresis of digested DNA: In the U.S.A. electrophoresis was conducted as described in publication #9, using a 20 x 20 cm horizontal slab gel electrophoresis apparatus (Pharmacia Model GNA 200) using TPE buffer. Gels contained 0.7% agarose, poured to a depth of 4 mm. Electrophoresis was carried out for 16-17 hr at 20°C. After electrophoresis the gels were stained with ethidium bromide and DNA bands visualized with UV light.

In Israel the digested DNA was electrophoresed at 30 V for 18 h in 0.8% agarose (Seakem; FMC Corp., Rockland, Maine) slab gels in buffer containing 0.04M Tris-HCl, pH 8.0, 0.005M sodium acetate, and 0.001M EDTA.

Genomic fingerprint analysis of Southern blots: DNA fragments from gels were transferred to NC sheets by the method of Southern (1975) and were hybridized with a probe consisting of plasmid pMC5, carrying the 5S, 23S, and part of the 16S gene of one of the two rRNA operons of M. capricolum (Amikam et al., 1982,1984). Further details of the method are found in Publication #11, which is included in this report. Hybridization was also carried out with the conserved gene probe tuf, as described in publication #15. Total DNA of M. synoviae was also used as a probe.

The probes were nick-translated (Maniatis et al., 1982) with deoxycytosine tri(³²P) phosphate (New England Nuclear Corp., Boston, Mass.) to a level of 2×10^8 to 4×10^8 cpm/ug of DNA, and hybridization with the DNA fragments on the NC sheets was done by a modification of the procedures described by Weinstock et al. (1978) and Pollack et al. (1980). To prevent nonspecific binding of DNA during hybridization, the sheets were soaked in 6 X SSC (1 X SSC is 0.15M NaCl plus 0.015M sodium citrate (pH 7.2)) containing 0.2% (wt/vol) each of polyvinylpyrrolidone (Sigma Chemical Co., St. Louis, Mo., PVP-360), bovine serum albumin (Sigma; fraction V), Ficoll (Sigma; type 400), and 100 ug of denatured salmon sperm DNA per ml (Sigma; type III) for 6 h at 56°C. Hybridization was carried out in the same buffer solution containing the labeled probe for 18 h at 56°C. The sheets were then washed at 56°C by soaking for 30 min with shaking in two changes of 2 X SSC plus 0.1% sodium dodecyl sulfate (SDS) followed by three changes in 0.5 X SSC plus 0.1% SDS and three additional changes in 0.2 X SSC + 0.1% SDS. After air drying, the NC sheets were exposed at -70°C to Agfa Gevaert X-ray film with an intensifying screen.

Selection of clones for use as probes: Probes were prepared according to the method of Hyman et al (1987), as described in detail elsewhere (publication #7). Total DNA of M. gallisepticum S-6 or M. synoviae WVU1853 was digested with HindIII. Fragments were separated on a 0.7% low-gelling-temperature agarose gel, and those larger than 3kb were ligated into plasmid pUC13 (Viero and Messing, 1982). Clones containing M. gallisepticum or M. synoviae DNA fragments were selected (Maniatis et al., 1982). Clones were screened to determine the size of the mycoplasmal DNA insert; clones containing the longest inserts (about 6kb) were chosen for further study.

Preliminary screening of clones was carried out by Southern hybridization of the M. synoviae clones to total DNA of M. gallisepticum and of the M. gallisepticum clones with total DNA of M. synoviae. Hybridization was carried out at 56°C and washing was performed under non-stringent conditions (Hyman et al., 1987) in order to maximize the detection of possible cross reactions. Four clones of each mycoplasma carrying the largest inserts and showing no cross reactions with the heterologous species were chosen for further testing. After further testing, one probe (pMG4) was selected for further studies with M. gallisepticum and another (pSN27) for M. synoviae.

Preparation of hybridization filters: Dot-blot hybridization with purified DNAs was performed as described elsewhere (Hyman et al., 1987). Hybridization to DNA of bacterial cultures or with samples from experimentally-infected chickens was carried out by applying samples to NC membranes (BA85, Schleicher & Schuell, Dassel, Germany or Nitroplus 2000, MSI, Massachusetts, U.S.A.). Nylon membranes (Nytran 0.45 μ m, Schleicher & Schuell) were also used, but were found unsatisfactory for clinical materials due to difficulties in applying the viscous samples. Release of DNA from the organisms was carried out by placing the nitrocellulose membrane in contact with a filter pad containing 0.5M NaOH + 1.5 M NaCl, followed by three successive 5 min exposures to filter pads containing 0.5 M Tris, pH 7.5 + 1.5 M NaCl. After air-drying the filters were washed twice in 2 X SSC for 15 min each time. When samples are pure mycoplasma cells (not clinical samples) the NaOH procedure could be carried out on the dot-blot apparatus. Other methods tried by us (including adding an equal volume of 1% SDS) were less effective than NaOH treatment.) After air-drying the filters are baked at 80°C for 2 hrs under vacuum. Nylon filters were either baked or exposed to UV light according to the manufacturers' instructions.

Hybridization of probe: Hybridization of DNA immobilized on NC filters was done according to procedures described by Maniatis et al (1982). The probes were labelled with 32 P using the Multi-prime DNA Labelling System kit (Amersham, U.K.). Two methods of hybridization were routinely employed. Comparison between the methods showed no difference in sensitivity or specificity, and choice was made for technical reasons only. Hybridization was carried out in sealed plastic bags at the required temperature with shaking for 3-5 hrs in pre-hybridization buffer and approximately 16 hrs in hybridization buffer with addition of probe at about 5×10^6 cpm/100 cm^2 filter.

1. Aqueous method: Hybridization was carried out at 58°C. Pre-hybridization buffer (0.1ml buffer/ cm^2) consisted of 6 X SSC; 5 X Denhardt's solution; 0.5% sodium dodecyl sulfate (SDS); 100 μ g/ml sonicated and denatured calf thymus DNA. Hybridization was carried out overnight in the same buffer (0.05 ml/ cm^2) with the addition of 0.01 M EDTA. Unless otherwise specified, post-hybridization washing was carried out with shaking as follows: 2 X 10 min each with 2 X SSC at RT; 1 h with 0.5 X SSC + 0.1% SDS at 58°C; 2 x 10 min each with 0.1 X SSC at RT.

2. Formamide method: Hybridization and prehybridization were carried out at 37°C, unless stated otherwise, in the following solution: 50% formamide, 5% dextran sulfate, 1M NaCl, 1% SDS and 100 ug/ml carrier DNA. Post-hybridization washing was carried out as follows: 2 X SSC + 0.1% SDS at RT for 10 min; 2 x SSC + 0.1% SDS at 37° for 30 min; 0.2 X SSC + 0.1% SSC at RT, twice for 15min.

Overnight autoradiography revealed the positive hybridization reactions when compared to positive and negative control spots containing M. gallisepticum and M. synoviae organisms respectively. Positive controls were included in 10-fold dilutions in order to have a quantitative estimate of effectiveness of hybridization.

Non-radioactive probe detection methods: Two methods were tested for non-isotopic labeling of probe pMG4:

1. Chemical modification of probe: The DNA probe was chemically sulfonated using the "Chemiprobe" kit (Orgenics, Yavne, Israel). Following hybridization according to the manufacturers' instructions the modified probe is detected by a monoclonal antibody reacting only with the sulfonated DNA. Color detection is with an alkaline phosphatase linked system.

2. Biotinylation of probe: Biotinylation was carried out by Random Prime reaction using Biotin-11-dUTP (BioRad, Richmond, CA) or Biotin-14-dATP (BRL, Bethesda, MD). Color detection was with the BioRad Biotin-Blot Clone Detection kit (Catalog No. 170-3556) or the BRL DNA Detection System (Catalog No. 8239SA), each according to manufacturer's instructions. Comparison with ³²P-labeled probe was made, using the standard hybridization methods described above.

Experimental infection experiments for evaluation of genetic probe: In Israel M. gallisepticum- and M. synoviae-free chicks (strain "Anak") were obtained from a commercial heavy breeder flock at 1 day of age, and maintained in a positive-pressure controlled-environment isolation cell. At time of inoculation birds were divided into experimental treatment groups and maintained in separate cells. A non-infected control group was maintained under the same conditions. The usual method for experimental infection was intra-tracheal inoculation but infection by fine-spray aerosol and intra-air sac inoculation were also employed. Contact infection of chickens was carried out by placing non-infected birds together with infected birds in a Horsfal-Bauer

isolation cell or by placing 1 or 2 "seeder" chickens in a M. gallisepticum-free group. Further details on experimental design are described elsewhere (publication #7, included in the Appendix).

Sampling for tracheal infection: Tracheal swabs from live experimentally infected birds or from the excised trachea of infected birds were suspended in sterile basal mycoplasma medium (BM). Isolation of mycoplasmas or titration to determine the number of viable mycoplasmas was carried out on this suspension prior to its application to NC filter. Disruption of mucus in tracheal swab suspensions was carried out with acetyl-cysteine (AC), as described in publication #7. For field studies, specimens (approximately 20 samples per flock) were collected by swabbing the trachea with a dry cotton swab which was transported in an ice box to the laboratory and processed within several hours. The swabs were vigorously suspended in BM (0.6 ml), and kept on ice until tested. If not applied immediately to filters, samples were frozen at -70°C. Aliquots of 0.1 ml of the suspension of tracheal material were applied to a NC filter using a dot-blot apparatus. Mycoplasma isolation and identification was carried out by standard methods.

RESULTS AND DISCUSSION

DNA relatedness and variation among species and strains

A. The use of probes to highly conserved genes for examination of intra-species variation: The use of cloned highly conserved mycoplasma genes as probes in Southern blot hybridization with digested mycoplasmal DNAs was exploited to test genetic homogeneity of the M. gallisepticum and M. synoviae species. The results are presented in full in publication #14, which is included in its entirety in this report (see Appendix).

A total of 34 M. gallisepticum and 7 M. synoviae strains were tested by Southern blot hybridization with the ³²P-labeled pMC5 plasmid carrying the highly conserved rRNA genes of M. capricolum. The resulting hybridization revealed a degree of genetic heterogeneity among the M. gallisepticum strains, more pronounced than that found for the M. synoviae strains. The hybridization patterns were found to be effective "genomic fingerprints", distinguishing strains within the M. gallisepticum species. As shown in Fig 2 of publication #14, the hybridization pattern of BglII-digested DNAs of M. gallisepticum strains fall into "clusters" of unique patterns of bands highlighted by reaction with the rRNA probe.

The most important finding in terms of application to epidemiology and diagnosis is the ability to differentiate the live vaccine F-strain from virulent field isolates of M. gallisepticum. Thus it was possible for the first time to definitely trace the origins of the F vaccine strains, and to test the premises that the F vaccine strain may displace resident virulent M. gallisepticum in areas where vaccination is carried out. Field isolates from F-strain vaccinated and non-vaccinated flocks in Israel were identified by this method. Moreover, field isolates from the collection of the Univ. of Georgia were tested. In some cases the identification of M. gallisepticum isolates as "F-strain" enabled epidemiology of a field outbreak to be clarified.

Similarly, several other strain clusters could be distinguished in M. gallisepticum by the fingerprinting technique. One group which could be recognized by a characteristic hybridization pattern is that of the so-called "variants" or atypical M. gallisepticum strains, which have similar biological properties (e.g., antigenic differences in serological tests, infectivity and reduced virulence). Three representatives of this group were tested.

The fingerprinting method with the rRNA probe enabled also the distinction of M. gallisepticum strains of unusual host origin. This was the first indication of the degree to which these so-called M. gallisepticum strains differ genetically from other representatives of this species, as has been shown more recently by DNA-DNA hybridization studies (J. Bradbury, personal communication) and infectivity properties (Yagihashi et al, 1988).

Our study also resulted in the development of a new conserved gene probe, the tuf gene, encoding for the prokaryotic elongation factor protein Tu. This protein fulfills an essential function in promoting the binding of aminoacyl-tRNAs to ribosomes and is, therefore, ubiquitous among prokaryotes.

We have recently cloned the tuf gene from M. pneumoniae and compared its nucleotide and deduced amino acid sequences with those of other tuf genes. The results (described in publication #15) revealed that the mycoplasmal tuf gene is highly homologous to that of other prokaryotes and, as such, can be regarded as a highly conserved gene. Apart from the importance of this conserved gene as a phylogenetic marker in studies of bacterial evolution, we could show that it may also be useful as a probe in taxonomy and epidemiology, resembling the conserved rRNA genes. Thus, patterns obtained by Southern blot hybridization of digested mycoplasmal DNAs with the cloned tuf gene as a probe, revealed genotypic heterogeneity among M. gallisepticum strains. Most interestingly, the M. gallisepticum strain clusters (including the F cluster) distinguished by the tuf gene probe, corresponded exactly with those distinguished by the rRNA gene probe pMC5. Further details of this study are summarized in publication #15.

The "genomic fingerprinting" approach appears to be of value in providing a new taxonomic tool enabling the determination of genetic homogeneity or heterogeneity within established species. Moreover, it may also be useful as a diagnostic and epidemiological tool, where identification of strains at the intraspecies level is required.

B. Use of restriction endonuclease analysis, polyacrylamide gel electrophoresis, and DNA probes in epidemiological studies of M. gallisepticum and M. synoviae: This approach was employed by the group at Univ. of Georgia. In these studies restriction enzyme digestion patterns were the basis of the comparisons made between various strains. Publications #8, 9, & 10 give further information relating to this study.

In the study reported in publication #10, restriction endonuclease analysis utilizing EcoRI was used to study M. gallisepticum field isolates. Strains from a series of outbreaks in North Carolina in 1980 and 1981 were compared. Turkey isolates from one major poultry producing area fell into 2 groups of related organisms; however, an isolate from a peacock that was suspected to be the source of the infection was unrelated to the turkey strains. Strains isolated from other geographic areas of North Carolina in 1985 were not related to the turkey isolates from 1980 and 1981, but one chicken strain and one turkey strain had identical restriction patterns. These same strains were compared antigenically using hemagglutination inhibition (HI). Antigenic relatedness by HI did not always correspond with the results of relatedness by restriction endonuclease analysis.

A series of isolates from a broiler breeder company in Pennsylvania was also studied. An isolate from 1986 was previously shown to have a restriction pattern similar to the F strain. However, M. gallisepticum strains isolated in 1987 and 1988 were not related to the F strain, but were similar to each other. Strains isolated in 1988 from turkeys in California were unrelated to the F strain or to any of the other cultures studied.

M. synoviae isolates from a multi-age commercial egg operation in Ohio were also compared. A strain isolated from a recent outbreak in a breeder flock was compared with several strains from the commercial layer operation located about 2 miles away. Restriction patterns of strains from the layer operation were similar, but were clearly different from the patterns of strains from the breeders, indicating that the commercial layers were not the source of infection in the breeders.

M. gallisepticum isolates from multi-age layers which had been vaccinated with live F strain vaccine were studied by restriction endonuclease analysis with EcoRI, SDS-PAGE, and with species-specific and F-strain specific DNA probes (Khan et al, 1987) to determine if the vaccine strain had displaced the original field strain on such farms. All recent isolates had restriction patterns similar to the parent vaccine strain and were positive with the species-specific DNA probe as well as with the F strain-specific probe. The original strain from one of the farms had a restriction pattern unlike the F strain, was negative with the F strain-specific probe, and was positive with the M. gallisepticum species-specific probe. SDS-PAGE results were more variable, but were in general agreement with the restriction endonuclease and DNA probe results.

The overall results of this work show the value of restriction endonuclease analysis in epidemiological studies. These results are also in agreement with studies using strain-specific and species-specific DNA probes as well as the results of genomic fingerprints. The restriction enzyme pattern analysis is more easily applicable than genomic fingerprinting, in particular since there is no necessity for radioactive labelling. However a possible limitation is the difficulty of comparison of patterns containing hundreds of bands and the detection and quantitation of minor band differences.

C. Genetic and antigenic relatedness between *M. gallisepticum* and *M. synoviae*. In order to answer the question as to whether the two avian pathogens are genetically related, both molecular genetics and immunoblot techniques were employed. The two mycoplasmas were subjected to Southern blot hybridization of digested DNA of one mycoplasma with total DNA of the other mycoplasma as a probe by a method developed by us (Yogev and Razin, 1986). Southern blot hybridization of *M. gallisepticum* A5969 DNA digested by *EcoRI* with the rRNA probe pMCS revealed three hybridization bands of 8.0 kb, 8.2 kb, and 9.0 kb, the pattern of which differed from the hybridization patterns of the rRNA genes for several other mycoplasmal DNAs digested with *EcoRI*. The use of nick-translated total DNA of *M. synoviae* as a probe in Southern blot hybridization with *EcoRI*-digested DNAs of the mycoplasmas tested revealed additional bands only with *M. gallisepticum* DNA. In all other cases the *M. synoviae* total DNA probe reacted only with the rRNA gene bands lighted also by the rRNA gene probe, so that the patterns obtained with the *M. synoviae* total DNA probe were identical with those obtained with the rRNA pMCS probe. Hybridization bands additional to those obtained with pMCS were observed also with *HindIII*- and *EcoRI*-digested DNAs of seven *M. gallisepticum* strains hybridized with total *M. synoviae* DNA as a probe, as shown in publication #17. The patterns obtained were different for the various *M. gallisepticum* strains. Thus the hybridization data revealed the sharing by the two mycoplasmas of genomic nucleotide sequences, additional to those of the highly-conserved rRNA genes.

The assumption that some of the shared sequences encode for antigens or epitopes common to both mycoplasmas was supported by Western blot analysis of cell proteins of one mycoplasma with specific antiserum to the other mycoplasma. The Western immunoblots (see publication #17) revealed antigenic

determinants common to M. synoviae and M. gallisepticum. The various M. gallisepticum strains differ in immunoblot patterns. Thus strains 4229 and 30902, isolated from a duck and goose, respectively, exhibited an immunoblot pattern which differed from that of the typical M. gallisepticum strains isolated from chicken (strains PG-31, A5969 and R). Similarly, the pattern of the "variant" M. gallisepticum strains (var.503 and var.730) appears to differ from that of the other two M. gallisepticum strain clusters in one of the two cross-reacting bands. The immunoblot patterns of M. synoviae strains produced with the anti-M. gallisepticum antiserum appear similar to each other, apart from that of strain FMT.

Interestingly, the differences in band patterns of reactive antigens among the M. gallisepticum strains supports again the concept that this species is genotypically heterogenous. Our results may explain the cross-reacting of the two mycoplasmas noted previously in a variety of routine serological tests, as will be discussed in the continuation of this report.

Serodiagnosis and immunogenicity of M. gallisepticum and M. synoviae

A. Comparison of ELISA, RSA, and HI tests: In the first part of this study (see publication #3), the sensitivity and specificity of commercial ELISA kits were evaluated and compared with the standard agglutination (RSA) and HI tests for M. gallisepticum. All RSA antigens proved to be highly sensitive when testing M. gallisepticum-positive sera. Laboratory-prepared RSA antigens yielded fewer positive reactions when testing M. gallisepticum-negative sera than commercial RSA antigens. Both M. gallisepticum ELISA kits showed high rates of positive reactions when testing sera from birds given commercial M. synoviae bacterin, fowl coryza (Haemophilus paraqallinarum) bacterin, or inactivated infectious bursal disease virus vaccine, and to a lesser extent fowl cholera (Pasteurella multocida) bacterin. Immunization with Frey's medium with 12% swine serum-in-oil or Staphylococcus aureus-in-oil resulted in sera which yielded numerous positive ELISA reactions. During the first 1 to 3 weeks, antibodies induced by experimental infection with M. gallisepticum were better detected by the RSA test than by the ELISA tests and the HI test, thus, confirming the RSA test's importance in mycoplasma diagnostic serology. The HI test can serve to confirm positive RSA results.

In a continuation of the study (details found in publication #4), two commercial ELISA tests, the RSA test, and the HI test for antibodies to M. gallisepticum were compared using a battery of antisera from M. gallisepticum-infected birds, M. synoviae-infected birds, or from birds given inactivated oil emulsion vaccines. The RSA test was the most sensitive. The HI test had the highest specificity. False positive reactions were seen in the ELISA tests and to a lesser extent in the RSA test.

B. Potential causes of false reactions in serological tests: Immunoblot analysis indicated that some commercial oil-emulsion poultry vaccines induce a strong antibody response in birds to various mammalian sera. Thus it appeared that poultry are being hyperimmunized to medium components which may affect ELISA serology for mycoplasmas and other avian pathogens that are cultured in serum-supplemented medium.

Antisera produced by aerosol infection of birds with M. gallisepticum, M. synoviae, M. gallinarum, or M. gallinaceum were used to detect the presence of immunogenic epitopes shared among these species. Chickens infected with M. gallinarum or M. gallinaceum produced very weak systemic antibody responses. Thus infection with these species, as is commonly found in layer chickens, should not cause a problem of false reactions in these serological tests.

Following infection with M. gallisepticum, 25 polypeptides from 30 to >200 kDa were immunogenic. Birds infected with M. synoviae responded to 11 polypeptides ranging from 17 to 179 kDa. Many proteins of M. gallisepticum and M. synoviae had common epitopes, the detection of which by immunoblotting was strongest in the first 4 wks following infection. This is approximately the same time post-infection (PI) that cross-reactions were seen in M. gallisepticum diagnostic tests using M. synoviae sera. Chickens hyperimmunized to Staphylococcus aureus responded with antibodies which recognized M. gallisepticum polypeptides of 156 and 108 kDa.

Marked antigenic heterogeneity of 6 M. gallisepticum isolates was evident when antisera to the R strain was used in immunoblotting. This antigenic heterogeneity was especially evident with the M. gallisepticum serological variants K503 and K703. These data allowed for the preliminary selection of M. gallisepticum immunogenic species-specific polypeptides of 85, 67, and 29 kDa to be tested as ELISA antigens.

C. The humoral immune response as measured by immunoblotting: The humoral immune response to M. gallisepticum was documented by immunoblotting. The strongest response occurred between 12 and 26 days post infection when 25 polypeptides were immunogenic. Cross-reactions and shared epitopes during this period were followed, as reported above.

In a continuation of this study (publication #5), the humoral immune responses of white Leghorn chickens, experimentally infected with M. gallisepticum or M. synoviae by an aerosol or contact exposure, were compared by immunoblotting. Details are presented in publication #5. Chickens infected with M. gallisepticum responded similarly regardless of mode of infection. A strong antibody response was seen in aerosol-infected chickens from 11d PI until the end of the 116d trial. M. gallisepticum-contact-infected chickens showed a more variable response with respect to time of appearance of antibody response and with respect to the pattern of bands developed. Chickens infected by aerosol of M. synoviae responded to more antigens and with greater intensity than did contact-exposed birds. Cross-reaction with proteins of M. synoviae was also seen in contact-infected birds, which would be presumed to be infected with M. gallisepticum organisms which were not grown in artificial medium.

Antisera from chickens from field infections with M. gallisepticum or M. synoviae were also analyzed by immunoblotting. There was no correlation between HI titer and intensity of immunoblot staining.

These antisera were used to identify species-specific protein bands of M. gallisepticum and M. synoviae. In addition to previously reported species-specific protein bands of M. gallisepticum, two highly immunogenic species-specific protein bands of 19 and 16 kDa were identified. Two species-specific protein bands of M. synoviae with relative molecular masses of 53 and 15 kDa were identified. Additionally, a highly immunogenic 34 kDa protein band of M. synoviae was identified. All protein bands identified in these mycoplasmas were purified by preparative SDS-PAGE in amounts sufficient for further characterization and for use in serodiagnostic tests.

D. Purification of immunospecific proteins: In this study, selected immunogenic protein bands of M. gallisepticum strain R and isolate F10-2AS of M. synoviae were purified from sodium dodecyl sulfate-polyacrylamide gels, as described in Materials and Methods. Purified M. gallisepticum protein bands of 67 (p67) kDa and 19/16 (p19/16) kDa, and purified M. syno-

viae protein bands of 53 (p53) kDa, 34 (p34) kDa, and 15 (p15) kDa were evaluated as potential ELISA antigens. Full details and figures of immunoblots are given in publication #6. Results of the dot-ELISA test are reported in the continuation of this report.

Improvement of test antigens

A. Sensitivity and specificity of *M. gallisepticum* antigens grown in medium with liposomes substituting for serum: A major cause of nonspecific agglutination with mycoplasma agglutination antigens is related to the presence of serum in the growth medium. Studies were conducted to improve the specificity of *M. gallisepticum* serum plate agglutination antigens without sacrificing sensitivity by growing the antigen in medium substituting artificial liposomes for serum. Various growth parameters were used in two different experiments, and antigens prepared in batch (static) culture were compared with antigens grown in a fermenter.

All batch-grown *M. gallisepticum* liposome antigens were highly sensitive and specific, while fermenter grown liposome antigens were insensitive. Antigen yield of fermenter-grown liposome antigens (grown in autoclaved medium) was lower than for other antigens. The yield per liter of liposome batch-grown antigen was related to the concentration of liposomes in the growth medium, whereas other growth parameters (such as incubation time and glucose concentration) had no effect on the yield. The reduced yield and sensitivity of antigens grown in a fermenter may have been due to autoclaving the medium instead of sterilizing it by filtration. Separation of mycoplasma cells from artificial liposomes was accomplished by centrifugation on a 25% (w/v) sucrose cushion. Separation of the mycoplasma cells from liposomes allowed the standardization of antigens by packed cell volume.

When compared to antigens prepared in Frey's medium with 12% swine serum (regular FMS) or to commercial RSA antigens, liposome antigens had a higher degree of specificity; however, they were similar in sensitivity and antigen yield. There was no obvious difference in SDS-PAGE patterns of S medium grown, L medium grown, or commercial RSA antigens.

The importance of the results of these studies is that we have shown that it is possible to propagate mycoplasma agglutination antigens in serum-free medium, thus significantly improving the specificity of such antigens. Results of these studies are given in publications #1 and #2.

B. Use of purified immunospecific proteins for dot-ELISA test: Chicken antisera produced by experimental and natural exposure to M. gallisepticum or M. synoviae, and by immunization with oil-emulsion vaccines were used to evaluate these antigens in a dot-ELISA test. M. gallisepticum antigen p67 detected antibodies in experimentally infected chickens 3 days after the RSA test and 7 days before the HI test (see Table 1). M. gallisepticum antigen p67 was able to detect antibodies to 12 M. gallisepticum isolates, including 2 serologically atypical isolates (K503 and K703).

Further tests were carried out on chicken serum sent to the Univ. of Georgia laboratory from suspected field outbreaks of mycoplasmosis. As shown in Table 2, these tests revealed that antigen p67 could detect natural M. gallisepticum infections. No cross-reactions with M. synoviae-positive antisera were seen with antigen p67. M. gallisepticum antigen p19/16 did not perform as well as p67. However, p19/16 may have some utility as an ELISA antigen if mixed with p67. M. synoviae antigens p53 and p15 did not perform well enough to be recommended as ELISA antigens. Antigen p34 of M. synoviae performed well with experimental antisera and with sera submitted from natural outbreaks of mycoplasmosis in chickens. The p34 antigen detected antibodies 5 days after the RSA test and at least 11 days before the HI test. However, some M. gallisepticum-positive antisera reacted with M. synoviae antigen p34.

The conclusion of these studies was that two antigens of M. gallisepticum (p67 and p19/16) and M. synoviae antigen p34 performed well enough to warrant further study as ELISA antigens. In particular, the p67 antigen of M. gallisepticum shows promise as a diagnostic antigen which will not give false positive reactions. The p34 antigen of M. synoviae also shows promise, but it has some cross-reactivity with M. gallisepticum.

In overall summary, these studies of serodiagnosis and immunogenicity reaffirmed the difficulties involved with nonspecific reactions in avian mycoplasma serology. Significant improvement in mycoplasma agglutination antigens was found by propagation of the organisms in serum-free medium. The antibody response to individual polypeptide antigens was studied and cross-reactive and specific antigens between M. synoviae and M. gallisepticum were identified. Potentially useful test antigens were further purified and evaluated in a dot blot procedure. The use of purified antigens in avian mycoplasma serology should eliminate false positive reactions resulting from antibodies in the test sera directed towards serum components. Future studies should concentrate on procedures for large scale purification of these antigens and their evaluation as serodiagnostic aids.

Preparation and testing of genetic probes:

A. Properties of the DNA probe: Four probes prepared from the DNA of M. gallisepticum S-6 and selected on the basis of preliminary screening were tested by the dot-blot procedure against the purified DNA of 29 strains of M. gallisepticum (10 ng/dot) and 9 strains of M. synoviae (100 ng/dot). Hybridization was carried out under non-stringent conditions in order to detect any possible cross-reaction between the two Mycoplasma species. Differences were seen in the intensity of the reaction between certain probes and some of the M. gallisepticum strains. For the present purpose a probe which reacted strongly with all the M. gallisepticum DNAs and showed no reaction with any M. synoviae DNA was selected; this was designated pMG4. Preliminary screening of the clones originating from the M. synoviae genomic library was carried out by Southern blot hybridization to total DNA of M. gallisepticum, as described in Materials and Methods. One of the clones selected was designated pSN27 and was used for further testing.

The sensitivity of probe pMG4 was estimated by reaction with purified homologous DNA (M. gallisepticum S-6) as shown in Fig 1 of publication #7 (included in its entirety, see Appendix). A clear positive reaction is seen with 250 pg DNA. No reaction with 100 ng M. synoviae DNA was seen. Fig 3B shows the reaction of probe pMG4 with M. gallisepticum organisms. The number of viable cells in the first dot-blot well was about 10^7 cfu; the M. gallisepticum probe detected 10^4 cfu.

The M. synoviae probe pSN27 reacted with the homologous type strain of M. synoviae and five other strains, including two local isolates (Fig 2, publication #7). Positive reaction was found with 10^5 - 10^6 cfu of all the M. synoviae strains tested. Test of probe pSN27 with isolated homologous DNA showed a reaction with 5 ng. Later studies have indicated that greater sensitivity vis a vis purified DNA is exhibited by M. synoviae clone pSN34, and it is recommended that further studies be carried out to investigate the use of this clone as a gene probe.

On testing post-log phase cultures, it was found that the decreased reactivity of the probes in detecting mycoplasmas in older cultures did not decrease proportionally to the decline in number of viable cells, but was usually 10 to 100-fold lower than the detection level to logarithmic viable cultures. No reaction of either probe was found with cultures of other avian mycoplasmas containing at least 10^7 cfu/sample or with the respective purified DNAs (1000ng). No reaction was seen with DNAs of other bacteria (E. coli, A. faecalis) found in the chicken trachea.

B. Testing of the genetic probe in experimental infection: An experimental infection model in which chickens were infected by intratracheal inoculation was used to test the effectivity of M. gallisepticum probe pMG4 in vivo. Details of experimental design and results are presented in publication #7 (see Appendix). The probe detected M. gallisepticum in samples taken by tracheal swabs of live chickens or from the excised trachea of necropsied birds. A positive hybridization reaction with pMG4 was found in most (but not all) infected birds during the first 2 weeks after infection, but thereafter an increasing percentage of samples appeared negative on testing with the DNA probe. Determination of the levels of viable mycoplasma in the trachea showed that tracheal levels of M. gallisepticum in infected chickens increase, peak and decrease with time after infection. The time course was not the same under all conditions, and variation between experiments was marked, probably due to differences in the severity of clinical signs. Most birds infected by intratracheal inoculation, and necropsied 4 to 23 days PI contained at least 10^4 CCU M. gallisepticum in tracheal suspensions, and often much more. Birds sacrificed later showed lower mycoplasma levels, with an increasing percentage of birds showing $<10^2$ CCU. Nonetheless, M. gallisepticum could usually be isolated after long incubation from the chickens containing low levels of mycoplasma, although in some cases the presence of secondary infections interfered with culture. In Table 2 of publication #7 are presented results of an experiment in which the amount of M. gallisepticum in the trachea is related to reactivity with the probe. Most birds showing tracheal levels of more than 10^5 CCU/ml M. gallisepticum reacted with probe pMG4, although at each time of testing only about half of the infected chickens reacted. Tracheal levels of mycoplasma in this experiment decreased markedly 12 days PI (relative to 7 days PI), which coincided with the clinical observation of accumulation of large amounts of mucus in the trachea. Tracheal swabs from non-infected chickens showed no hybridization with probe pMG4; testing of a cross-section of highly M. gallisepticum-positive samples with the M. synoviae probe also showed no reaction.

The ability of the M. gallisepticum probe to detect early stages of infection is described in detail in publication #7. In order to approximate the situation in natural infection under field conditions, contact-infected chickens were tested. In this case serological activity (RSA and HI) is the indication for the presence of infection and progression of disease. Fig 3 in publication #7 shows further results of one such experiment. The samples shown in this figure were taken at one sampling time, 17 days PI for

air sac-inoculated chickens and 13 days for contact-exposed chickens, thereby representing different stages of infection. The ability of the probe to detect M. gallisepticum appears to be directly related to the amount of organisms in the tracheal swab suspension. Only chickens containing at least 10^4 CCU in the dot on the hybridization filter gave a positive reaction with pMG4. In spite of RSA activity and diagnostically-significant HI titers not every air sac-infected chicken gave a positive reaction with the probe. Thus, serological activity was not necessarily related to positive reaction with probe.

One of the major problems encountered in application of DNA probes to clinical material is the physical consistency of specimens. In order to overcome the technical difficulties encountered in processing tracheal samples containing mucus, treatment of the samples with acetylcysteine was attempted, as described in publication #7. This treatment improved the effectivity of the probe in detecting mycoplasma, especially at later stages of infection. A strong hybridization reaction was also found in tracheal swab specimens from carcasses of M. gallisepticum-infected chickens for which no other method is available for testing is available due to massive post mortem contamination.

Application of the gene probe method under field conditions

A. Comparison of the gene probe with conventional diagnostic methods: At the same time as probe pMG4 was being tested in experimental infection, samples from known field outbreaks of M. gallisepticum were examined. Identification of flocks to be tested was based on the standard methods of serological testing (RSA and HI), and were brought to our attention by field practitioners. Methodology for sampling was in accordance with usual field procedures for testing breeder flocks, e.g., tracheal swabs from live birds. Particular emphasis was placed on simplicity of laboratory manipulations, with an eye for future applications in diagnostic laboratories.

Tracheal swab samples from a suspected flock were tested in parallel by two procedures: (1) isolation and identification of mycoplasmas; (2) hybridization with probe pMG4. In early tests we attempted to perform both procedures on the same tracheal swab or on two tracheal swabs taken simultaneously from the same bird. At a later stage tracheal swabs were tested either for hybridization or for isolation; comparison of the effectivity of the methods was made between two groups of birds rather than on an individual basis. Serological tests were carried out in parallel. In particular, we

were interested in the comparative effectivity of the methods and the relative time required for obtaining results.

An additional use of the gene probes was in rapid identification of the enrichment cultures. In this case a color-change in the mycoplasma growth medium may be due to M. gallisepticum, but may also be attributable to M. synoviae or any of several rapidly-growing saprophytic avian mycoplasmas. The method for distinguishing between these possibilities is by growth on solid media and identification by IMF staining, requiring an additional 4-7 days. The probe could be used for economical and rapid testing of a large number of suspicious cultures, without the necessity to wait for growth on agar. Since the amount of mycoplasma DNA was usually quite high in the cultures, only a intermediate level of sensitivity is required for the probe, which may enable the use of a hybridization protocol somewhat shorter than that used for clinical material.

Results of the use of the probe for direct diagnosis of M. gallisepticum in four field outbreaks, encompassing five flocks (two of which were tested twice) are reported in publication #11. Results are summarized separately for chicken and turkey flocks due to differences in clinical details.

Table 3 shows the results of diagnosis of M. gallisepticum infection in two outbreaks in chicken breeder flocks. The presence of infection was indicated during routine monthly serological screening. No clinical signs of infection were noticeable and birds were sampled at random. In the case of the flocks from Kibbutz 1 (line 1) and Moshav 4, Farm S (lines 2 and 3), serological results were clearly indicative of M. gallisepticum infection as was verified by reaction with probe pMG4 and, in due course, by isolation. However, the flock from Farm G on Moshav 4 (located close to Farm S) showed positive DNA hybridization reactions in direct tracheal swabs even though results of serological testing were not diagnostically significant at that time. Cultivation of tracheal specimens from all the chicken flocks indicated the presence of several other species of mycoplasma, interfering with M. gallisepticum cultivation and producing mixed cultures as evidenced by IMF staining. The use of probe pMG4 for rapid identification of M. gallisepticum in culture was particularly effective in these cases.

The effectiveness of probe pMG4 in diagnosis of M. gallisepticum infection in turkey flocks is also reported in publication #11. Typically for turkeys, the first indication of infection came from clinical signs of respiratory distress. Thus, in contrast to the situation in chicken flocks, at least some of the turkeys sampled were showing signs of infection. Due to secondary bacterial infections of the respiratory tract, isolation of M.

gallisepticum in such cases was carried out with difficulty. In each of the three flocks tested there was a clear indication of the presence of M. gallisepticum in direct tracheal swabs, even though serological results were not diagnostically significant at the time of sampling. With time, M. gallisepticum infection was confirmed by the development of high HI titers. The dot-blot hybridization results of the tracheal samples from naturally infected turkeys are shown in Fig. 1. Samples from the choanal cleft of the same birds showed no positive reaction with the probe, and neither did swabs from the sinus cavity and exudate, although these sites are sometimes recommended for M. gallisepticum isolation (Branton et al, 1984). There was no indication of reaction of probe pMG4 with other bacteria present at high levels in these samples. Duplicate filters and duplicate spots on the same filter are useful to increase confidence in results.

B. The use of probe pMG4 for early detection of infection: The effectivity of the probe in detecting early stages of infection, as was found in the experimental infection model, was borne out under field conditions. Two field cases were examined where the probe was applied to flocks which showed no serological evidence of M. gallisepticum infection. These flocks were tested only because of their close proximity to diagnosed M. gallisepticum outbreaks. These results are reported in publication #12.

Field Outbreak 1: Two flocks of 5 month old broiler-breeder chickens were maintained in adjacent units, separated by a two meter aisle. About two weeks after detection of positive reactions to M. gallisepticum in the RSA test, M. gallisepticum infection in the larger flock (9500 birds) was confirmed by positive reactions with the DNA probe pMG4 and by diagnostically significant HI titers. Serological sampling of the adjacent flock (50 samples from 5000 birds) showed no positive reactions in the RSA test, although a clear positive reaction was seen with the M. gallisepticum probe. With time in each flock, M. gallisepticum was isolated from about the same proportion of samples as were positive in the hybridization reaction, confirming the level of infection indicated by the DNA probe. Ten days later (at time of slaughter) only 3/25 samples showed a detectable RSA reaction.

Field Outbreak 2: At time of monthly serological screening suspected positive reactions in the RSA test were detected in two out of 7 broiler-breeder flocks (7 months old) located in conventional open buildings separated from each other by about 10 meters. Confirmation of the M. gallisepticum break

was obtained by HI results when tested 10 days later, at which time the DNA probe gave positive hybridization with tracheal swab suspensions from each bird tested. Probe hybridization of tracheal swab samples (12-16 each) of the other flocks in the complex indicated that M. gallisepticum infection was detectable at various levels in each one. However, serological results showed only 9/25 and 3/25 positive RSA reactions in two flocks, and no positive reactions in the other 3 infected flocks. Isolation of M. gallisepticum from each of the flocks supported results with the DNA probe.

The basis of the gene probe method of diagnosis is the specific reaction with DNA of the agent, unrelated to the serological response of the host. Thus success is dependent in the first place on the number of organisms in the specimen tested. As has been shown in experimental infection models (Levisohn et al, 1987; publication #7) tracheal levels of M. gallisepticum rapidly reach levels which are within the detection range of the M. gallisepticum probe. In the absence of interfering circumstances, isolation of M. gallisepticum is also readily accomplished at this stage of infection, as was found in these field outbreaks. However, a complicating factor in these flocks was the presence of M. synoviae. This agent causes uncertainty in serological tests, and complicates isolation and identification. Under other circumstances, or together with M. synoviae, one or several species of saprophytic mycoplasmas may be found in the trachea of chicken breeders, making cultivation of M. gallisepticum virtually impossible.

The significant observation in these studies is that under field conditions the probe may detect M. gallisepticum in chickens 1-2 weeks before a serological response is detected. Rapid detection and elimination of infected flocks should be useful in confining the outbreak and minimizing losses.

C. Field application of the M. synoviae gene probe: In spite of repeated attempts, the use of the M. synoviae probe for hybridization with M. synoviae in tracheal swabs under field conditions has not been successful in direct sampling of tracheal swabs. It is assumed that this is related to the lesser sensitivity of probe pSN27 relative to that of probe pMG4 (at least 10-fold less). Another possible explanation is that tracheal levels of M. synoviae do not reach the levels found for M. gallisepticum. Further field testing of M. synoviae probes will be necessary before it is possible to come to a conclusion about applicability in this system.

D. Non-radioactive labeling of probes: Within the framework of this project a major objective was to test the feasibility of the gene probe concept of diagnosis in the avian system. In doing so, we have focussed on the question of feasibility of using the system giving the best chance of detection--at this stage, ^{32}P label. If the principle is successful adaption to a non-radioactive label may be carried out as a later development, exploiting the state-of-the art method applicable at that time. This is not to imply that we feel that the radioactive method will find a wide acceptability. In addition to the problems connected directly with the use of ^{32}P there is the problem of the short half-life of the probe, making it virtually impossible to do any quality control.

Tests were carried out with the Chemiprobe method of chemical modification of the pMG4 probe. Using the methods reported for the M. pneumoniae gene probe, as reported by us (Hyman et al, 1987), only limited success was found with the M. gallisepticum probe. With modifications of the protocol by the manufacturers', some improvement was seen in the detection of purified DNA and cultures of M. gallisepticum. Under the best conditions the sensitivity of the Chemiprobe approached that found with ^{32}P . Test of clinical samples was, however, negative. Problems were encountered with non-specific color development and lack of reproducibility. The procedure is multi-staged and time-consuming.

Better results were obtained with the biotinylated probes. Sensitivity was equivalent to that found with ^{32}P . Only preliminary experiments were carried out comparing the methods with each other and with ^{32}P . It should be noted that the hybridization method (aqueous or formamide) may influence the color development in the biotin-detection system. Moreover, according to the manufacturer's (BRL) instruction manual) the T_m of the biotinylated probe differs from that of the non-biotinylated DNA. It will be necessary to critically test the temperature and time of hybridization, as well as the recommended concentration of probe, to find optimal conditions for this system. It is clear, however, that the use of biotinylated probes will considerably shorten the detection time. The biotinylated probe was successfully used for rapid identification of mycoplasma cultures from field outbreaks. The method was not tested with M. gallisepticum-positive direct tracheal samples.

Table 1. Detection of seroconversion in M. gallisepticum inoculated^a chickens with the M. gallisepticum Rapid Slide Agglutination, Hemagglutination Inhibition and Dot-ELISA tests.

Days PI	RSA ^b		HI ^c	DOT-ELISA ^d	
	PDRC	INTER		p67	19/16
0	0/10*	0/10	0/10	0/10	0/10
6	1/10	2/10	0/10	0/10	0/10
7	2/10	6/10	0/10	0/10	0/10
9	9/9	8/9	0/9	7/9	3/9
12	10/10	10/10	0/10	10/10	5/10
16	10/10	10/10	5/10	10/10	5/10

^aChickens were inoculated intraocularly and intranasally with approximately 10^7 color changing units of M. gallisepticum strain R.

^b Rapid slide agglutination antigen; PDRC=laboratory prepared, INTER= commercial antigen (Intervet, Holland).

^c Hemagglutination Inhibition test using M. gallisepticum strain A5969, as described in M & M. All positive HI titers were 1:40.

^d Dot-ELISA test as described in M & M; p67=67 kilodalton (kDa) protein band of M. gallisepticum strain R; p19/16= 19 and 16 kDa protein bands. Both antigens were purified from SDS-PAGE gels.

* Number positive sera/ number sera tested.

Table 2. Ability of M. gallisepticum (Mg) and M. synoviae (Ms) purified antigens to react in a dot-ELISA with chicken antisera from natural field outbreaks of mycoplasmosis^a

Case	Mycoplasma isolated ^b	RSA ^c +	HI ^c +	Diagnosis ^d	DOT-ELISA ^e			
					Mg		Ms	
					p67	p19/16	p53	p34
89-1009	NT	NT	Ms	Ms	0/4*	0/4	NT	2/4
89-1015	NT	NT	Ms	Ms	0/4	0/4	1/4	3/4
89-1013	NT	NT	Ms	Ms	0/4	0/4	0/4	1/4
K2847	NT	NT	Ms	Ms	0/7	0/7	NT	3/7
89-1056	B,D,Mg	Mg,Ms	Mg	Mg	5/13	3/13	0/10	0/13
89-1200	B,D,Mg	Mg,Ms	Mg	Mg	5/12	2/12	0/8	0/12
K2786	Mg	Mg	Mg	Mg	3/4	2/4	0/4	0/4
K2833BC	Mg	Mg,Ms	Mg	Mg	8/12	5/12	0/4	1/12
K2818D	B,D	Mg,Ms	Mg	Mg	5/5	0/5	0/5	0/5
K2846	B	None	None	B	0/5	0/5	NT	0/5
K2848	B	None	None	B	0/5	0/5	NT	0/5

^aAntisera and in some cases tracheal swabs were submitted to the Univ. of Georgia laboratory for mycoplasma diagnosis.

^b B= M. gallinarum; D= M. gallinaceum. Mycoplasmas isolated identified by fluorescent antibody test.

^c Rapid slide agglutination(RSA) and Hemagglutination Inhibition (HI) antigen for Mg and Ms respectively were from strains A5969 and WVU1853 respectively; HI titers of >1:40 were positive.

^d Diagnosis based on serology and culture results available.

^e Antigens purified from SDS-PAGE as described in M&M and text.

^f NT=Not tested; either serology or culturing was not done.

* Number of sera positive/number tested.

Table 3. Use of DNA probe pMG4 for diagnosis of M. gallisepticum in field outbreaks in chicken breeder flocks

<u>Flock^A</u>	<u>Serology^B</u>	<u>Identification of Mg</u>		
		<u>Direct^C</u> <u>Probe</u>	<u>Culture^D</u> <u>Probe</u>	<u>IMF</u>
1. Kibbutz 1	100% RSA + HI+++	4/22 ^E	20/22 ^E	7/9 ^E
2. Moshav 4, Farm S	100% RSA + HI +	12/18	Not tested	
3. Moshav 4, Farm S*	100% RSA + HI +	9/19	14/19	4/5
4. Moshav 4, Farm G	40% RSA + HI -	7/20	18/20	6/8

^A Flocks were heavy breeder chicken flocks, age 8 months (Kibbutz 1) and 10 months (Moshav 4).

^B Rapid Slide Agglutination (RSA) and Hemagglutination Inhibition (HI)

tests were performed according to standard methods (U.S.D.A., 1985)

Serological tests (Kibbutz 1, 100 samples out of 14000 birds; Moshav 4, 20 samples out of 2500 birds, each flock) carried out one month earlier showed no positive reactions in the RSA test.

^C Direct examination of swab material.

^D Culture of material from swab.

^E Number of positive samples/number of samples tested.

*Tested 3 days after first sampling time.

Figure 1. Dot-blot hybridization of tracheal samples from a turkey breeder flock. Autoradiogram of dot-blot filter hybridization of samples from suspensions of tracheal swab contents taken from a flock suspected of *M. gallisepticum* infection. Samples 1-12 were taken from individual turkeys; samples from the trachea and choanal cleft of the same birds were placed in parallel wells. Positive hybridization with *M. gallisepticum* organisms and no hybridization with *M. synoviae* organisms are indicated.

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