PN-ACP-706
114850

FINAL REPORT
Covering Period: Jan. 1, 1996 -Nov. 30 2000

Submitted to the U.S. Agency for International Development
Bureau for Global Development
Center for Economic Growth

AVIAN MYCOPLASMOSIS BIOTECHNOLOGY APPLIED IN PERU

Principal Investigator: Dr. Sharon Levisohn
Kimron Veterinary Institute, Bet Dagan Israel

Project number: C11-220
Grant No. TA-MOU-95-C11-220

A.I.D. Grant Project Officer: David O'Brien

Project Duration: Jan. 1, 1996-Nov. 30, 2000

2. Executive Summary

Control of the major avian pathogens *Mycoplasma gallisepticum* and *M. synoviae* is a keystone of the modern poultry industry due to the synergistic effects of these infective agents with all major poultry pathogens (bacterial and viral) and exacerbation of the infections under intensive growth conditions. Strengthening the productivity and improving the self-sufficiency of the poultry sector of the Peruvian agricultural economy is important in order to provide an economical source of animal protein.

The project addressed these concerns in several ways. The main operational goal was to provide a framework for transfer and application of technology for state-of the art molecular diagnostic tests (PCR) for *M. gallisepticum* and *M. synoviae*, developed and validated by project collaborating scientists. This was accomplished by establishing a Molecular Biology Unit in the Poultry Department at San Marcos University. The PCR tests are part of a full range of diagnostic tests for the pathogenic mycoplasmas. This provided, for the first time, reliable information on the health status of various poultry sectors in Peru, and guidelines for control programs. On-going development within this project of the cost-effective, time-saving generic PCR tests, and particularly validation under field conditions, has also lead to their widespread acceptance in mycoplasma diagnostic laboratories in the USA, Europe and Israel. The Molecular Biology Unit in Peru is economically self-sufficient on a cost-fee basis.

The project has been highly visible, as manifested in part by the large number of workshops and courses initiated by the Peruvian scientists, in which the CDR-funded project was clearly acknowledged. An ongoing network of international connections has been established, growing out of visits by the project collaborators and other mycoplasma experts in Peru and project-funded participation of the Peruvian scientists in major international meetings. The project fostered local research initiative, which is responsive to concerns of the Peruvian poultry industry, helping to build bridges between university scientists and the poultry industry, for the benefit of both.

2. Table of Contents

1. Cover Page 1
2. Executive Summary 2
3. Table of Contents 3
4. Research Objectives 4
5. Scientific Report 5
   - Introduction 5
   - Materials and Methods 5
   - Results and Discussion
     - I. Field Studies (Peru) 6
       - Evaluation of mycoplasma status of layer flocks 6
       - Efficacy of drug treatment on persistence of mycoplasma 7
       - Production parameters in MG vaccinated layers 7
     - II. Laboratory Studies (Peru) 8
       - Adaptation of PCR for detection of mycoplasma in embryos, pipped eggs and progeny chicks 8
       - Relationship between maternal antibody, MS status and health status of progeny 9
       - Clearance of MS from the upper respiratory tract by local treatment with antibody and tiamulin 9
     - III. Laboratory studies and trials (Israel) 10
       - Application of MG-PCR for diagnosis of MG and *M. imitans* in geese 10
       - Evaluation of the risk of natural *M. imitans* infection in chickens 10
       - Investigation of "non-specific" PCR reactions in breeder flocks 10
       - Application of PCR tests for avian species other than domestic poultry (free-flying birds) 11
     - IV. Use of MG-PCR and MS-PCR for diagnosis (Peru and Israel) 11
   - Tables and Figures 12-23
6. Impact Relevance and Technology Transfers 24
7. Project Activities/Outputs 24
8. Project Productivity 30
9. Future Work 30
10. Literature Cited 31

4. Research Objectives:

The overall goal of the project was to develop biotechnology-based diagnostic tests for pathogenic avian mycoplasmas, and to apply these as the basis of an integrated control strategy for mycoplasmosis in poultry flocks in Peru. A major operative goal was to establish a unit with capability for molecular biotechniques (particularly PCR) and standard diagnostic tests, as part of the existing Poultry Laboratory at San Marcos University, headed by project collaborator Dr. E. Ichocea. The rationale for the location of the Mycoplasma PCR unit in this department was to exploit the existing framework in which diagnostic testing for other poultry pathogens is carried out on a fee/cost basis. We envisioned this unit as a prototype for other LDC's. The project furthermore aimed to produce a core of scientists and technicians conversant in the relevant biotechniques. Fostering an independent self-sustaining research capacity that is responsive to local (Peruvian) issues is an important step in this direction. Another essential aspect has been to increase the awareness of the importance of mycoplasma and to build confidence and acceptance by veterinarians in governmental regulatory agencies and commercial companies.

The basic premises of this project are:

1) Strengthening the productivity and improving the self-sufficiency of the poultry sector of the Peruvian agricultural economy is important in order to provide an economical source of animal protein.

2) Control of the major avian pathogens *Mycoplasma gallisepticum* and *M. synoviae* is a keystone of the modern poultry industry due to the synergistic effects of these infective agents with all major poultry pathogens (bacterial and viral) and exacerbation of the infections under intensive growth conditions.

Initial support for establishment of the Molecular Biology Unit came from within the university itself. The strengthening and expanding of the pre-existing Poultry Department, in spite of major economic constraints, manifested this policy. Support from the poultry industry, directly from large grower companies or through their professional organizations, has been steadfast. Commercial companies (vaccine and anti-microbials) consult with the collaborating scientists, participate in joint field studies and exploit the network of international connections. The Molecular Biology/Mycoplasma Diagnostic Unit, based on the equipment and expertise provided by this grant, is now economically self-sufficient.

5. Scientific Report

Introduction

Data presented in previous Annual Scientific reports:

1. Development of new primers for mycoplasmas (Auburn)
2. Modifications of procedures for preparation of template for PCR (KVI and Auburn)
3. Field evaluation of PCR tests (KVI)
4. Adaptation of method for testing synovial fluid (KVI)
5. Comparison of Auburn PCR tests to IDEXX commercial PCR test in experimentally infected chickens (Auburn) and to other generic PCR systems (KVI)
6. Integration of PCR methodology in mycoplasma control programs (KVI and Peru)

The data in this report relate to the research areas for which data were not presented previously, or in which additional information is now available. The formal collaboration of Dr. Lauerman (Auburn) was finished at the end of 1998, when he retired from Auburn University. The no-budget extension of the project in 1999 and 2000 was for continuation of experiments in Peru. During the extension, project associated research in Israel was carried out on a limited scale, funded by other research grants. This was in order to provide scientific support for the research in Peru. Project- associated budget for Israel during the extension period was utilized for an advisory visit by Dr. Levisohn in Peru (1999).

Materials and Methods:

Polymerase Chain Reaction (PCR): The MG-PCR and MS-PCR tests were performed with primers MG-13/14 and MSL-1/2 with amplification according to Lauerman (1998). Use of primers MG1/2 and restriction enzyme *Vsp* I to differentiate between *M. gallisepticum* and *M. imitans* was according to Kempf (5).

Serological tests: Serological testing was carried out according to the protocols recommended in the National Poultry Improvement Program (NPIP) manual (1). The Rapid Slide Agglutination (RSA) test was performed with specific stained antigen (Intervet, Boxmeer, The Netherlands), according to manufacturer's instructions. The ELISA test was carried out with commercial ELISA kit (Kirkegaard & Perry Laboratories), according to manufacturer's instructions.

Isolation of Mycoplasmas: Isolation and identification of *M. gallisepticum*, *M. synoviae* and other avian mycoplasmas was by standard methods (13), with identification by immunofluorescence with specific antisera.

Collection of data: Samples for field studies and surveys were collected by project-initiated and project-funded visits by the collaborating scientists, research assistants or students to the farms. Referred diagnostic samples were received from company or poultry service veterinarians, at their initiative. In Israel most samples tested are from chicken or turkey breeder flocks, and are submitted for testing due to results of preliminary serological tests results, which indicate the possibility of mycoplasma infection. Follow-up or repeat testing for investigative purposes is at the initiation of the Mycoplasma Unit.

Animal infection trials: All animal trials in Israel are carried out under the supervision of the Animal Welfare Committee of the Kimron Veterinary Institute.

Results and Discussion

I. Field Studies (Peru):

Evaluation of mycoplasma status of layer flocks:

A study of seroprevalence of mycoplasmosis in twelve commercial layer farms in Chincha was carried out during the period May until December 1998. Sampling was done with sera from 300 hens from different strains and ages. Sera were analyzed by enzyme-linked immunosorbent assay (ELISA) and Rapid Slide Agglutination (RSA) tests, for *Mycoplasma gallisepticum* (MG) and *M. synoviae* (MS). Of the 300 sera tested for MG, 117 were positive by RSA, and 147 by ELISA; 51 sera were positive for MS by RSA and 260 by ELISA. Prevalence values were estimated

by ELISA because RSA showed low sensitivity, probably due to the chronic nature of the infection. The RSA test detects primarily IgM, which has a relatively short half-life. The prevalence of MG and MS was 49% and 88,64% respectively. Corrected prevalence was 47% (+ 0,082%) for MG and 88,67% (+ 0,055%) for MS. **These serological results show that commercial layers from Chincha are infected with MG and MS, with a high proportion of coinfection. Furthermore, it is recommended that seroevaluation be based on the ELISA test.**

In a follow-up study, the hemagglutination inhibition test (HI) and an indirect ELISA (KPL) were used to detect antibodies against MG and MS from 102 commercial layer hens at Chincha Province, Ica - Perú . The HI test detected antibodies to MG in 20 % of the layers tested whereas the ELISA detected 52%. The reactors for MS were 5 to 95 % by HI and ELISA, respectively. The polymerase chain reaction (PCR) was set up as a reference test to estimate the sensitivity, specificity, predictive positive value, and predictive negative value to the serological tests HI and ELISA. The ELISA test was more sensitive for detection of antibodies to MG (67 %) and MS (97 %) than the HI test for MG (12 %) and MS (5.3%). The specificity recorded for ELISA, relative to the PCR test results, was 68 % to MG and 25 % to MS in comparison to 70.4% and 100 % specificity recorded by HI for MG and MS respectively. Using the Kappa index test we can determine that there is no concordance between HI and ELISA for the diagnosis of MG and MS. Furthermore, the Mc Nemar test shows that HI can not replace ELISA. **These results indicate the necessity for further correlative studies of serology vis á vis PCR.**

A comprehensive study was made of layer flocks located in the central coast region of Peru where most commercial layer farms are concentrated. The hen's ages were from 26 to 70 weeks old. Clinical records of the selected flocks indicated previous mycoplasma outbreaks, drug treatment to mycoplasma and no vaccination. Serum samples were taken from 176 layers in 14 flocks to test for serological reactions to MG and MS by Rapid Slide Agglutination and ELISA (KPL). Trachea and choanal cleft samples from the studied 14 flocks were taken by swabs to examine the presence of MG-DNA and/or MS-DNA by PCR assay. Pools of three or five samples were tested for PCR of both Mycoplasmas.

Eight and nine of the flocks were positive to DNA-Mg for trachea and choanal cleft, respectively. On the other hand, 13 and 11 of the 14 flocks were MS-DNA positive by PCR assay of trachea and choanal cleft. Differences among RSA, ELISA and PCR results (Table 1) probably reflects that the studied flocks had been suffered from acute to chronic forms of the mycoplasma infection during the sampling stage. The relative sensitivity of the tests used varies according the disease stage (12).

All layer flocks in the present study were found infected by MG or/and MS by antibody or/and DNA-PCR criteria. The distribution of the infected flocks is displayed in Table 2. Ten of the 14 flocks were positive by PCR and RSA or/and ELISA; 7 of the infected flocks were positive by all three assays. The remaining 4 flocks were diagnosed as MG infection only by presence of antibodies against MG by RSA or/and ELISA. MS infection in the studied flocks showed similar picture to MG infection. Thirteen of the 14 flocks were positive to DNA-PCR and RSA or/and ELISA; 9 of them were positive to the three tests; only one flock was recognized as MS infected only by the serological results in the ELISA test.

By considering the overall mycoplasma status in the study, a MG/MS co-infection was recognized in 9 of 14 flocks by PCR. Four flocks were only diagnosed as MS infection; only one flock was negative to both MG-PCR and

MS-PCR, but this flock positive by serological tests (Table 2). **According to the mentioned results, the presence of MG/MS co-infection in layer flocks in the central coast region of Peru, diagnosed by PCR and serology, was found more frequently than previously known, a finding which has major significance for the growers in this region.**

Efficacy of drug treatment on persistence of mycoplasma:

M. synoviae is an economically significant pathogen of commercial poultry worldwide, which generally produces subclinical to mild upper respiratory tract or synovitis infections with lateral and vertical transmission. The effect of tiamulin hydrogen fumarate treatment on agent persistence in the trachea of MS infected layers was evaluated. Two groups of eight, thirty week old , MS infected (PCR positive) commercial layers (Isabrown line) were studied. The first group received two three day treatments of tiamulin (33mg./kg administered in the drinking water) with a 27 day interval, while the second group was not medicated. Tracheal swabs were taken weekly for two months. **All 16 samples were tested by PCR, using pools of four tracheal swabs, and found positive to MS confirming pathogen persistence in the trachea of both treated and untreated birds.** These results are in agreement with those reported by Kempf et al (7), but as pointed out by those authors the PCR-positive samples may reflect the presence of non-viable DNA.

Production parameters in MG vaccinated layers:

A field study was conducted to evaluate the production parameters in a laying flock vaccinated with the *Mycoplasma gallisepticum* (MG) ts-11 strain and a marginal economic analysis was performed to compare the results 9,065 free-MG laying pullets were placed on a previous detected MG positive farm. Birds were divided in two groups and placed in two separated sections in the same house. The vaccine was given to one of the groups at three weeks old and the other group remained non-vaccinated. Serologic tests; serum plate agglutination (RSA), hemagglutination inhibition (HI), enzyme linked immunosorbent assay and PCR were used to determine challenges by MG, MS and infectious bronchitis virus; *Salmonella enteritidis was monitored by culture.* Serum antibody response provoked by the MG vaccine was measured by RSA and ELISA. Vaccinated hens laid 188 eggs/hen and 11.55 kg egg/hen over a 41 week production period compared with 184 eggs/hen and 11.34 kg/hen in non vaccinates. Feed conversion rate was 2.57 and 2.62 for the vaccinated and the non vaccinated group, respectively. There was significant difference (p less than 0.05) in the production parameters evaluated and the marginal economic analysis showed better results for the vaccinated group.

II. Laboratory Studies (Peru):

Adaptation of PCR for detection of mycoplasma in embryos, pipped eggs and progeny chicks:

A series of experiments were carried out to investigate the possibility to detect embryo-transmission from mycoplasma infected breeder flocks in the progeny. Following up on preliminary experiments carried out in Israel (Ramirez and Levisohn, data not shown), it was decided to test for the presence of MG in pipped eggs. It has been previously reported (8), and confirmed in our laboratory, that the prevalence of MG in pipped eggs, as detected by culture, is much greater than in randomly selected one-day old chicks, presumably because the MG weakens the embryo, and the chick does not succeed in hatching.

The presence of MG-DNA was tested in different tissues of pipped chicks and embryos from hens with natural infection and experimental infection respectively, using PCR. In a preliminary experiment, 60 pipped chicks and 60 1-day old chicks from a naturally infected breeder flock were negative to MG-DNA and MS-DNA by PCR, from trachea, palatine cleft and thoracic airsacs (Table 3). An experimental inoculation with MG, strain K 810 was performed in order to prove different infected tissues in embryos through the detection of DNA-MG. MG-DNA was detected from yolk sac in embryos from 13 to 21-day old (Table 4).

The finding of MG-DNA by PCR was done in forty five pipped chickens from infected breeder flocks, obtained from yolk sac, esophagus, trachea and allantoic membrane (Table 5). All the esophagus pools (5/5) and three yolk sac pool were positive for MG. Only one allantoic membrane pool was positive for MG. All the trachea pools were negative for MG. These same samples were negative for MS. All the samples were confirmed for MG by culture obtaining the same results. **These results clearly indicated that the esophagus is the recommended tissue for PCR testing of embryos.**

The presence of maternal antibodies anti-MG and antibodies anti-MS were shown in nineteen chickens of one-day-old, nine of them hatched normally and ten were weak (Table 6). An ELISA commercial kit (KPL) was used to evaluate maternal antibody levels in both groups of chicks. Seven samples were seropositive to MG in each groups. All the sera were negative to MS. There was no correlation between the detection of antibodies anti-MG and antibodies anti-MS by ELISA's test and rapid slide agglutination. Comparison of ELISA titers in healthy and weak chicks indicated significantly higher levels in the healthy birds (Fig 1). This is in agreement with previous results (10) that indicated that maternal antibodies may reduce MG levels in ovo, and protect against the embryo pathogenicity of MG infection. It was not possible to find MG-DNA in one-day-old chicks, in either esophagus or yolk sac. **It is concluded that the ability to detect MG infection is greater in dead-in-shell embryos and pipped chicks than in hatched chicks.**

Relationship between maternal antibody, MS status and progeny health:

In an additional study, 120 21 day embryos and forty -five chickens originating from the same breeder flock placed in Lima and diagnosed as positive to MS , were evaluated in order to find MS-DNA by PCR in different organs and to titrate serum antibodies against MS. No MS-DNA was found in the chick samples, while in the 21 day old embryos a relatively low passage level was detected (2/18). No statistical association between the animal condition and the presence of mycoplasma by PCR was found. Esophagus was the only organ that has a diagnostic significance, confirming earlier studies. This work showed a lower maternal antibody titer to in the embryos than in the chickens, which was associated with the smaller vitelic absorption. Comparison of antibody levels of MS in dead-in-shell embryos and 1-day old chicks showed, in three separate trials, that the ELISA titers are higher in healthy hatched chicks than in the embryos (Table 7). This does not appear to be related to the amount of unabsorbed yolk, as seen in Table 8. Vertical transmission of MS seems to be very low in natural conditions and incubation problems are suspected to be mutifactorial, and not necessarily related to MS transmission.

Clearance of MS from the upper respiratory tract by local treatment with antibody and tiamulin (Peru)

Various authors (12,13) have reported that efficacy of antibiotic treatment is decreased in animals that have an impaired immune response. It has been

suggested that clearance of MS from the respiratory tract of infected animals may be more effective in the presence of specific antibody together with presentation of anti-mycoplasmal drug. A laboratory trial was designed to test this hypothesis. A flow chart for the experiment is shown in Fig. 2, showing 3 successive treatments with anti-MS antiserum and tiamulin by aerosol. Presence of MS was followed by MS-PCR at times post-treatment, as indicated. **MS was found at all times of testing and the combined antibody/antibiotic treatment had no detectable effect on clearance of mycoplasma from the trachea.**

III. Laboratory studies and trials (Israel):

Application of MG-PCR for diagnosis of MG and *M. imitans* in geese:

As previously reported, the Auburn MG-PCR test is directed to a segment of the 16S rRNA gene that is >99% homologous in MG and the closely related avian mycoplasma, *M. imitans* (3). The sensitivity of primers MG13/14 is the same for MG and M. imitans. These organisms may be differentiated by use of an MG-PCR-RFLP tested based on primers MG1/2 (6), or by isolation in specialist laboratories with appropriate immuno-reagents. *M. imitans* has been isolated from natural infection in ducks and geese in Europe and Israel but never been identified in the USA, probably due to the problems in differential identification. MG is not usually considered a pathogen of domestic water fowl although it has been isolated from these hosts (15).

MG has been identified as the causative agent in clinical respiratory disease in young geese (15). Molecular typing of the MG isolates shows a clear connection with MG strains isolated from outbreaks of disease in chicken and turkey breeder flocks. The MG-PCR and MG-PCR-RFLP tests are used for monitoring MG and *M. imitans* infection in goose flocks in Israel. *M. imitans* is frequently found in adult breeder geese, with no clear cut clinical manifestations (2). Until now, there is no evidence for the presence of asymptomatic MG infection in geese.

Experimental infection of goslings with MG or *M. imitans* clearly demonstrated the pathogenic potential of both species (Table 10). Both species, individually or in mixed infection, were diagnosed in disease lesions. There is a strong correlation between results with the MG-PCR and MG-PCR-RFLP tests and isolation of MG or M. imitans. The feasibility of differentiation of the two species by the molecular techniques was clearly demonstrated and is preferable to standard culture techniques requires specialized techniques and reagents that are not generally available. Serological tests were not suitable for diagnosing MG in geese.

Evaluation of the risk of natural *M. imitans* infection in chickens:

M. imitans has not been reported in natural infection in chickens or turkeys. Nonetheless, the organism can colonize these hosts in experimental infection, and in synergism with viral agents can have clinical manifestations (3, 4), and the possibility of its presence under field conditions can not be dismissed. Furthermore, experimental infection with *M. imitans* has been reported to cause sporadic "non-specific" reactions in standard serological reactions used for monitoring MG in breeder flocks.

In order to test the effect of *M. imitans* on diagnostic tests (PCR and serology), an experimental infection trial in chickens was performed. Chickens were infected with *M. imitans* reference strain 4229 or a local *M. imitans* isolate from goose, ATS-7. As shown in Table 11, *M. imitans* colonized the chickens for at least 50 days. The organism was detected by MG-PCR from tracheal samples with sensitivity similar to that of isolation. Sporadic transient serological reactions in tests

for MG were found. The conclusion of the experiment was that *M. imitans* infection in chickens may cause non-specific reactions in the MG-PCR test.

Investigation of non-specific PCR reactions in breeder flocks:

It has been demonstrated that MG-PCR or MS-PCR may detect MG or MS infection prior to detection of specific antibodies. It has been proposed that the PCR tests be used for monitoring of breeder flocks prior to movement between regions, or spiking males before introduction to resident breeders. In addition, testing of breeder flocks from which fertile eggs or chicks are exported has been suggested.

In the course of MG surveillance several flocks demonstrated persistent low level serological reactions in the RSA test. MG-PCR tests found low frequency, low level positive reactions. The intensity or frequency of these reactions did not increase with repeat testing, as is usually found in MG infections. With time, the reactions in MG-PCR "disappear", that is to say, are no longer detected.

In order to clarify the nature of the "non-specific" reactions, we sequenced the MG-PCR amplicon, and compared to that amplified by reference strains of MG and M. imitans (Table 12). The gene segment is highly homologous, but not identical, in both reference species. The DNA amplified from the field case shows a high homology to both species, but differs in several base pairs. DNA alignments were made of other samples, with similar results. The results eliminate the possibility of laboratory contamination in the PCR tests since the product differs from the other organisms with which we work. In one of the field cases a reaction was found in the IDEXX MG-PCR test, supporting the presence of a non-pathogenic variant MG strain, but this was not confirmed by isolation. **The conclusion of the sequence analyses is that the purported "non-specific" MG-PCR reactions in breeder flocks are authentic reactions, due to the presence of mycoplasma DNA, probably a variant strain of either of the two above species.**

Previous results in the framework of this project demonstrated a non-specific reaction of the MG-PCR reaction with primers MG13/14, which was attributed to DNA of *Chlamydia* spp. present in certain flocks of breeder turkeys. Infection of turkeys with high tracheal levels of *Chlamydia* spp. is relatively infrequent, and has not been diagnosed in the USA. The amplicon resulting from the non-specific reaction differs slightly in size from the authentic reaction, and can be differentiated by electrophoresis or by sequencing of the amplified gene fragment.

Application of PCR tests for avian species other than domestic poultry (free-flying birds):

It has been suggested that transmission of mycoplasma by free-flying birds may be one of means by which MG or MS is introduced to breeder flocks maintained in stringent limited access biosecurity. The possibility of the organism being transmitted by various birds captured in the poultry house environment was examined over a period of several months. Reoccurring outbreaks of MG or MS had been diagnosed in the region, and in the particular sites. Table 13 shows a cross section of the birds examined. Tracheal samples from all I birds were tested by MG-PCR and MS-PCR, and in some cases culture was attempted. Amplification was found in five out of the eight samples tested. In one case there was a strong PCR product with the MS-PCR test. Sequencing of the amplicon, and comparison with GeneBank sequences of the target gene confirmed that the product was amplified from MS. Two samples were suspected to be MG, on the basis of the size of the amplicon, but the product was not sufficient for sequencing. The presence of MG was not confirmed by culture. Two other samples showed amplification of a gene fragment differing in size from the specific PCR product. In one case there

mycoplasmas was isolated, but was not identifiable with the reagents at our disposal. The results indicate that the **MG-PCR and MS-PCR tests may be valuable in *identification of these organisms in non-poultry avian species.*** A recent report (16) has shown the value of PCR for testing environmental samples, including wild birds, confirming our use of the test for this purpose. **However the results must be interpreted with caution, due to the possibility of *reactions with exotic* mycoplasma species for which the test has not been validated.**

IV. Use of MG-PCR and MS-PCR for diagnosis (Peru and Israel)

Table 14 is a compilation of PCR tests performed in the Biotechnology Unit at San Marcos University in 2000, including diagnosis and research. The numbers represent pooled samples, usually 3 tracheal swabs per PCR sample.

Table 15 is a compilation of PCR tests in the Mycoplasma Unit of Kimron Veterinary Institute during the first six months of 2000. The data represent only samples submitted for diagnosis within the framework of the mycoplasma control of breeder flocks. Since most Broiler-Breeder and Leghorn-Breeder flocks in Israel are chronically infected with MS, chickens are tested only for MG. Turkey flocks infected with either MG or MS will be slaughtered. Most flocks were tested as the result of suspicious or positive reactions in serological screening tests. In some cases the flocks were tested due to clinical signs (turkeys) or proximity to a prior MG outbreak, as a precautionary move. Several meat-type turkey flocks were tested to confirm vertical transmission from an infected breeder flock. Isolation was performed only upon request, or for research purposes. There is complete agreement between MG or MS isolation and PCR results. All MG isolates were typed for epidemiological studies.

Table 1: Correlation between Serological Tests and PCR to MG and MS in 14 Peruvian Layer Flocks

Mycoplasma		RSA	ELISA	PCR	
		Pos	Pos	Trachea	Choanal
MG	(n = 14)	11/14	12/14	8/14	9/14
MS	(n = 14)	9/14	14/14	13/14	11/14

Table 2: **DISTRIBUTION OF THE INFECTED PERUVIAN LAYER FLOCKS ACCORDING TO THE DNA OR/AND ANTIBODY DETECTION**

ANTIBODY/ DNA STATUS	**MG**	**MS**
	n = 14	n = 14
RSA - / ELISA - / PCR -	0/14	0/14
RSA - / ELISA - / PCR +	0/14	0/14
RSA - / ELISA + / PCR -	1/14	1/14
RSA + / ELISA - / PCR -	1/14	0/14
RSA + / ELISA + / PCR -	2/14	0/14
RSA + / ELISA - / PCR +	1/14	0/14
RSA - / ELISA + / PCR +	2/14	4/4
RSA + / ELISA + / PCR +	7/14	9/14

Table 3: DETECTION OF DNA-*Mycoplasma gallisepticum* (DNA-MG) AND DNA-*Mycoplasma synoviae* (DNA-MS) BY PCR IN PIPPED CHICKS FROM TWO BREEDER FLOCKS.

N° OF CHICKS	DNA	EXAMINED TISSUE FOR DNA-MG AND DNA-MS	
		TRACHEA (n = 12)	CHOANAL CLEFT-AIR SAC (n = 12)
60 CH	MG	0/12	0/12
	MS	0/12	0/12
60 PE	MG	0/12	0/12
	MS	0/12	0/12

( ) In parenthesis is displayed the number of examined "pool" from 60 chicks.

TABLE 4: RECOVERY OF DNA-*Mycoplasma gallisepticum* (MG-DNA) BY PCR IN EXPERIMENTAL INFECTED EMBRYOS.

INOCULUM	AGE (DAYS) OF THE EXAMINED EMBRYO	TISSUE EXAMINED FOR DNA-MG		
		TRACHEA	ESOPHAGUS	M. VITELO
C I	5-7-12	Neg.	Neg.	Positive
C IV	12	Neg.	Neg.	Neg.
C I 10-1	4-5	Neg.	Neg.	Positive
C IV 10–1	4-5	Neg.	Neg.	Neg.
C I 10-2	4-5	Neg.	Neg.	Neg.
C IV 10-2	4-5	Neg.	Neg.	Neg.
Sol. Saline	4-5	Neg.	Neg.	Neg.

TABLE 5: DETECTION OF DNA-M. gallisepticum (MG-DNA) AND DNA-M. synoviae (MS-DNA) BY PCR IN NO BORN CHICKS FROM A NATURAL INFECTED BREEDER FLOCK

No. OF CHICKS	DNA	CONDITION	EXAMINED TISSUE FOR MG-DNA AND MS-DNA			
			Trachea (n=5)	Esophagus (n=5)	M. Vitelo (n=5)	M. Alantoide (n=2)
45	MG	DIRECT	0/5	5/5	3/5	1/2
		CULTURE	0/5	5/5	3/5	1/2
45	MS	DIRECT	0/5	0/5	0/5	0/2
		CULTURE	0/5	0/5	0/5	0/2

( ) In parenthesis is displayed the number of "pool" examined

TABLE 6: DETECTION OF DNA AND ANTIBODIES *OF Mycoplasma gallisepticum* (MG) AND *Mycoplasma synoviae* (MS) IN ONE-DAY CHICKS FROM A NATURALLY INFECTED BREEDER FLOCK.

CHICK DETECTION CONDITION	Mycoplasma	DNA by PCR		ASSAY FOR ANTIBODY			
		Esophagus	M.Vitelo	RSA Positive	ELISA Positive	Susp.	Neg
Healthy (9)	MG	Neg.	Neg.	8/9	7/9	2/9	0/9
	MS	Neg.	Neg.	2/9	0/9	0/9	9/9
Sickly (10)	MG	Neg.	Neg.	8/10	7/10	3/10	0/10
	MS	Neg.	Neg.	3/10	0/10	0/10	10/10

Table 7: COMPARISON OF THE *Mycoplasma synoviae* ANTIBODY TITER OF DEAD-IN-SHELL EMBRYOS AND ONE-DAY CHICKS FROM A NATURAL INFECTED BREEDER FLOCK

Group	No	Antibody by ELISA					%CV
		+	±	-	Min.-Max. Titer	Average±SD	
I-Chicks	10	9	0	1	1732 –12425	5651± 4143	55.9
I-Embryos	29	1	9	6	820 – 3783	1020 ± 97	61.3
II-Chicks	10	10	0	0	3347 – 12425	6817± 3156	31.3
II-Embryos	28	22	3	3	799 - 8078	2099 ± 2105	67.8
III-Chicks	10	1	0	0	785 – 5277	2731± 1 546	41.6
III-Embryos	29	16	7	6	756 - 5 595	1521± 1 642	74.5

Table 8: ASSOCIATION BETWEEN *Mycoplasma synoviae* ANTIBODY TITER TO AMOUNT OF UNABSORBED YOLK IN DEAD-IN-SHELL EMBRYOS AND ONE-DAY CHICKS FROM A NATURALLY-INFECTED BREEDER FLOCK

Group	No. Pos	Antibody by ELISA		%CV	Yolk (ml) Avg ± SD
		Min-Max Titer	Average±SD		
Chicks (15)	14	1 007 – 5 927	4 507± 3 695	57.9	8.3 ± 1.9
Embryos (28)	16	786 – 7 314	1 951±2 562	95.4	5,0±1,0

All tissues examined for Ms-DNA were negative

Table 9: Anti MS effect by aerosolization of Tiamulin and MS-serum in MS infected hens, detected by PCR

	PCR				
Group	Days after last treatment				
	0	2	7	15	22
T	+	+	+	+	+
S	+	+	+	+	+
C	+	+	+	+	+

Table 10: Experimental Infection of Geese with *Mycoplasma gallisepticum or M. imitans*

Time post-infection	10-11 D PI			17 D PI			22-25 D PI			31 D PI 40 D (Groups I, IV)		
	RSA*	Isolation	PCR**	RSA	Isolation	PCR	RSA	Isolation	PCR	RSA	Isolation	PCR
Group I Control				1/ 3	1/ 1 MG	2/ 2				1/3	1/ 2MG 1/ 2 MIm	
Group II ATS-7 Aerosol	1/ 8 + 2/ 8 +/-	2/ 2 Tr MIm 2/ 2 AS MIm	8/ 8 Tr 2/ 2 AS	0/ 2	1/ 2 Tr MIm 0/ 2 AS	2/ 2 Tr 2/ 2 AS	0/ 4		2/ 2 Tr 1/ 2 AS	0/ 2	0/ 2	2/ 2 Tr 2/2 AS
Group III MG-R Aerosol	2/ 10 + 8/ 10 +/-	2/ 2 Tr MG 2/ 2 AS MG	10/ 10 Tr	1/ 8 + 2/ 8 +/-	NT	2/ 2 Tr 0/ 2 AS		2/ 4 Tr MG 1/ 4 Tr MG + MIm		0/ 2	0/ 2	0/ 2
Group IV MG-R IT	5/ 12 + 2/ 12 +/-	2/ 3Tr MG 1/ 3 Tr MG+Mim 2/3 ASMG 1/3 MIm	3/ 3 Tr 3/ 3 AS	1/ 9 + 1/ 9 +/-	1/ 1 Tr MG	1/ 1 Tr 1/ 1 AS						
Group IV-A Uninfected In contact		1/1 Tr MG 1/1 AS MG+ MIm	1/ 1 Tr 1/ 1 AS		1/ 1 Tr MIm 1/ 1 AS MG	1/ 1 Tr 1/ 1 AS		1/ 1 Tr MG 1/ 1 Tr MIm	2/ 2 Tr	0/5	3/5 Tr MIm	0/3 Tr

* Serological results in RSA test are + or +/- (delayed reaction), No./No tested.
Tr= tracheal swab; AS = air sacs; Mg= *M. gallisepticum*; Mim = *M. imitans*
** PCR from individual tracheal swabs in live birds or swabs from air sacs in birds sacrificed for testing.

Table11: Experimental Infection of Chickens with *Mycoplasma imitans*

Time post-infection	12D				21D				33D				50D	
	Serology**			Isolation /PCR*	Serology			Isolation /PCR*	Serology			Isolation /PCR*	Serology	Isolation /PCR*
	MM	MG	MS		MM	MG	MS		MM	MG	MS		MM MG MS	
Group I Control				Negative				0/2				0/2		
RSA		Negative												
Immunocomb		Negative											Negative	
Group II 4229 AS + T				1/ 2 PCR				3/ 4 PCR 3/ 4 M.im.				2/2 PCR		1/ 2 PCR 2/4 M.im.
RSA	0/4	4/4	4/4		NT	3/4	3/4		NT	0/4	0/4		Negative	
Immunocomb	4/4	3/4	3/4		4/4	0/4	0/4		NT	0/4	0/4		Negative	
Group II ATN-7 T only				2/2 PCR				0/4 PCR 3/ 4 M.im.				0/2 PCR		NT
RSA		Negative				Negative				Negative			Negative	
Immunocomb		Negative				Negative				Negative			Negative	
Group III ATN-7 AS + T				2/2 PCR				1/5 +/- 4/5 M.im.				0/2 PCR		NT
RSA	0/5	5/5	5/5		NT	3/5	2/5		NT	2/5	0/5		Negative	
Immunocomb	5/5	2/5	1/5		5/5	1/5	0/5		1/5	0/5	0/5		1/5 +/-	

* Pooled samples (2 or 3) unless otherwise indicated.
** Serological results in RSA test are + or +/- (delayed reaction), No./No tested. Low level reactions in immunocomb are indicated as negative.

Table12:
Comparison of sequences of PCR amplicon with primers MG13/14 (16S rRNA gene)

```
                                ▼
S-6SL   1     GGTTTTAGAT ATTACCAG-C TCTCATAGTT TGACGGGCGG TGTGTACAAG
4229SL  1     GGTTTTAGAT ATTACCAG-C TCTCATAGTT TGACGGGCGG TGTGTACAAG
BHSL    1     GGTTTTAGAT ATTACCAGAC TCTCATAGTT TGACGGGCGG TGTGTACAAG

S-6SL  51     ACCCGAGAAC GTATTCACCG CGACATGGCT GATTCGCGAT TACTAGTGAT
4229SL51      ACCCGAGAAC GTATTCACCG CGACATGGCT GATTCGCGAT TACTAGTGAT
BHSL   51     ACCCGAGAAC GTATTCACCG CGACATGGCT GATTCGCGAT TACTAGTGAT


S-6SL 101     TCCGACTTCA TGAGGGCGAA TTGCAGCCCT CAATCCGAAC TGAGACCAAC
4229SL101     TCCGACTTCA TGAGGGCGAA TTGCAGCCCT CAATCCGAAC TGAGACCAAC
BHSL  101     TCCGACTTCA TGAGGGCGAA TTGCAGCCCT CAATCCGAAC TGAGACCAAC

                   ▼
S-6SL 151     TTTACAG-TT TAGCTCA
4229SL151     TTTACAGTTT TAGCTCA
BHSL  151     TTTACAGATT TAGCTCA
```

Mycoplasma strains tested: S-6SL = *M. gallisepticum* S-6(reference strain);
4229SL= *M. imitans* type strain 4229; BHSL=PCR-amplified fragment, species unknown.

Sequence determined with forward primer, MG13.

Table 13: Presence of pathogenic mycoplasmas in free-flying birds in proximity of poultry house

COMMON NAME	LATIN NAME	PCR TEST RESULTS	CULTURE
Coot	*Fulica atra*	+++ MS	Not Tested
Rock Pigeon	*Columba livia*	Negative	Not Tested
Falcon	*Falco tinnunculus*	Non-specific amplification	Negative
Plover	*Vanellus spinosus*	Suspect MG	Negative
Palm dove	*Streptopelia senegalensis*	Suspect MG	Not Tested
Common dove	*Streptopelia turtur*	Negative	Not Tested
Pigeon	*Columba livia*		Not Tested
Crow	*Corvus corone cornix*	MS-PCR Non-specific amplification	Two types unidentifiable mycoplasma

Table 14: Polymerase Chain Reaction Test (PCR)*

Type of Birds	*Mycoplasma gallisepticum*		*Mycoplasma synoviae*	
	MG Negative	MG Positive	MS Negative	MS Positive
Layers	21	1	15	47
Breeders	43	2	31	79
BB Chickens	4	0	34	3
Turkeys	2	0	1	1
Cocks	1	0	0	1
Synovial Fluid	0	0	2	0
TOTAL	71	3	83	131

Data(in pools): Avian Pathology Laboratory (Biotechnology Unit) School of Veterinary Medicine.
San Marcos Univerisity
Year 2000

* From the report submitted to the VITAR (IVITA) January 2001

Table 15: Diagnostic PCR Tests performed in the Mycoplasma Unit, Kimron Veterinary Institute: January through June, 2000

Type of Bird	No. of cases/ samples	MG-PCR		Isolation[1]	MS-PCR		Isolation
		-	+		-	+	
Turkey[2] Breeders	18 flocks 590 TS	3	8 51/101	3/3	2	5 44/58	2/2
Meat[2] Turkeys	3 flocks 90 TS		3 30/30	1/1	NT	NT	
Broiler-breeders[2]	30 flocks 1186 TS	16	12 113/118 2 ±[3]	6/6	NT	NT	
Leghorn Breeders	1 flock 60 TS		1 ±[3]		NT	NT	

[1] Isolation was performed only when specifically requested, in PCR positive flocks..

[2] Samples from turkeys were tested only for MG or MS, according to request of veterinarian. Only MG-PCR was tested in chicken breeder flocks since most flocks are chronically infected with MS.

[3] Flocks that gave suspicious reactions (±) were further tested, as described in the text.

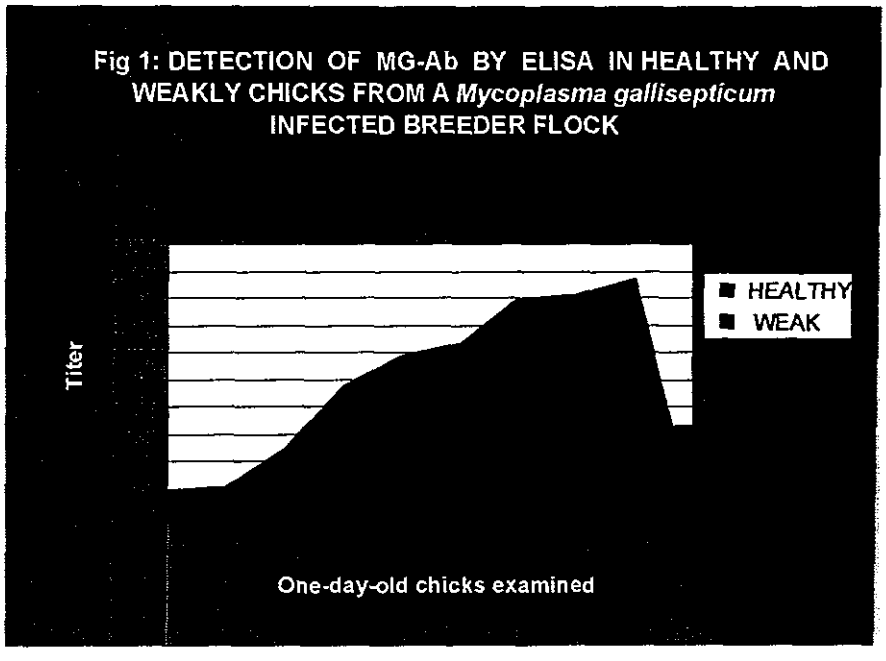

FIG. 2:
EXPERIMENTAL DESIGN OF THE ANTI-MS EFFECT BY AEROSOLIZATION OF MS-SERUM AND TIAMULIN IN MS INFECTED HENS

GROUP (N= 10)	TREATMENT Day 0	2	4	DETECTION OF MS-PCR Days post treatment 2	7	15	22
MS SERUM	----------	----------	----------	----------	----------	----------	----------
TIAMULIN	----------	----------	----------	----------	----------	----------	----------
SALINE	----------	----------	----------	----------	----------	----------	----------

All treatment were applied three times, in a 3 m^3 glass cabinet

Fig. 3:Biotechnology Unit : Avian Pathology Laboratory

Fig. 4: Part of the research team with bird visitors, that were transported alive from Chincha Province (at Ica Department) to Lima. Samples were taken from choanal cleft, eye and trachea.

6. Impact Relevance and Technology Transfers

The Molecular Biology Unit in the Poultry Department of San Marcos University is an economically self-supporting body within the university. The biotechniques introduced within the framework of this project are routinely performed on a cost-fee basis. In addition, the Poultry Department performs the standard serological diagnostic tests for avian mycoplasmas. The head of the department, project collaborating scientist Dr. E. Icochea, is a recognized poultry expert and consultant, not only in Peru but also in other Latin American countries and the laboratory serves as a reference center for several poultry diseases.

The Auburn MG-PCR and MS-PCR are widely accepted, in large part due to the validation studies carried out within the framework of this project. The reliability of the tests is firmly established and documented. Several mycoplasma specialist laboratories in the USA and Europe have introduced the test as a diagnostic option, in parallel or as an alternative to the commercial PCR test. The generic Auburn tests are highly cost-effective and time-saving, which are important factors for diagnostic applications. Additional innovative tests developed by Dr. Lauerman at Auburn University were not transferred to project collaborators.

As can been seen by the project activity described in Section 7 (meetings, workshops, short courses and students in Peru) the impact of the project was disproportionate to the level of funding. The various project-activities reached a large cross-section of veterinarians, microbiologists, poultry practitioners and growers. On all these occasions the accreditation to AID-CDR was prominently attributed and displayed.

7. Project Activities:

Visits of collaborating scientists:

1) Visit of Dr. Levisohn at Auburn University: A two day visit in March 1996 was devoted to discussions regarding plans for the impending visit of Dr. Lauerman to Peru, and general issues regarding long range planning. This was also an opportunity for observing the PCR laboratory set-up and protocols, with relationship to the recommendations for the Peru PCR unit. This visit was not budgeted for in the original project, but was made during the time that Dr. Levisohn was present in the USA for other purposes.

2) Visit by Dr. Lauerman in Peru: This visit, in April 1996, had three main goals:

a) DISCUSSIONS WITH KEY INDIVIDUALS IN THE UNIVERSITY AND POULTRY INDUSTRY: These took place throughout the visit. In addition to formal introductions for acquaintance purposes and detailed planning sessions, a short-course on avian mycoplasmosis was organized as part of the continuing education program of SM university. This was an all-day program of lectures and discussions attended by about 60 veterinarians. The course encompassed a review of the problem of avian mycoplasmosis, diagnostic methods (emphasis on molecular diagnostic methods) and strategies for control.

b) DEMONSTRATION OF PCR TECHNIQUE: A highly successful demonstration of PCR detection of *M. gallisepticum* was performed for approximately 25 faculty, graduate students and staff in attendance. This was the first time that

that MG-PCR was carried out in Peru. and that a PCR test was performed in the SM Vet. School.

c) VISITS TO POULTRY OPERATIONS IN PERU: A program, arranged by a major Peruvian breeder companies, included a visit to one of the breeder flock premises about 150 miles from Lima. Discussions were held with company veterinarians and officials about mycoplasma control and plans for future collaboration were discussed.

3) Training of Dr. Icochea at Auburn University: This was carried out during a period of one month in fall 1996. Most of period was spent in the laboratory of Dr. Lauerman at Auburn University, but included 3 days with Dr. Levisohn (on sabbatical at North Carolina State University), 2 days with Dr. J. Giambrone in the Poultry Science department, AU and trips to farms and breeder operations in Alabama. The main emphasis was on the performance of the PCR technique, which Dr. Icochea learned to perform independently. One major goal of the USA visit was to make decisions about equipping the PCR unit in the Peruvian laboratory. Significant amounts of small equipment and supplies were purchased through Auburn University and shipped to Peru for project purposes. Dr. Noe purchased a computer for the project while in the USA, and shipped it to Peru. Books and scientific journals needed for the project were also purchased in the USA. These purchases saved a lot of project money.

4) Training visit of Dr. Ramirez: In July-August 1997 Dr. Ramirez visited the collaborating laboratories in the USA (1 week) and Israel (4 weeks). During the time in Israel he made extensive visits to farms and breeder companies. Plans and training for joint research projects were carried out.

5) Visit of Dr. Levisohn at Auburn University: In May 1998 a 2 day visit was devoted to scientific updates and policy planning with respect to publication of results and continuation of the project. In addition there was exchange of samples and reagents for collaborative studies. Specific plans were made for the upcoming visit of Dr. Lauerman in Israel, in order to enable preparation and preliminary tests for the experiments to be carried out at that time.

6) Visit of Dr. Lauerman in Israel: In June 1998 Dr. Lauerman visited Israel for about 10 days, which included participation in the 10th European Poultry Conference in Jerusalem. Several experiments were carried out in the laboratory at KVI.

7) Visit of Dr. Levisohn in Peru: In Sept. 1998 Dr. Levisohn spent 3 weeks at San Marcos University, with project funding. In addition to on-hands experiments in the laboratory, there were field trips for sampling clinical material and visits on farms. Consultations and advisory sessions were held with students engaged in project-associated research. Dr. Levisohn presented a field-oriented lecture at a special meeting organized for veterinarians and professionals associated with commercial growers and companies supplying vaccines and drugs to the poultry industry. Important meetings were held also with the head of the Peruvian Poultry Association and representatives of the government regulatory agencies. Within the university several meetings were held with Dean Juan Espinso, the Research Authority for this project.

8) Visit of Dr. Levisohn at San Marcos University: In Sept. 1999, following participation in the poultry congress in Lima, Dr. Levisohn spent one week in the Poultry Dept. of San Marcos University with an agenda similar to that detailed above for the 1998 visit.

Joint participation in scientific meeting:

1) Dr. Noe, Dr. Icochea and Dr. Levisohn attended the IXth Congress of the International Organization for Mycoplasmology (Orlando FA) and participated in the meeting of the Avian Mycoplasma working team of the International Research Program in Comparative Mycoplasmology which were held in conjunction with the IOM. Dr. Levisohn introduced them to key scientists working in the field.

2) Dr. Noe, Dr. Icochea, Dr. Levisohn and Dr. Lauerman attended the meeting of the American Association of Avian Pathologists (AAAP) in Louisville KY in July 1996. The AAAP is the most important USA meeting in the area of poultry diseases. In addition to the many relevant scientific presentations there was also a project strategy meeting.
3) Dr. Ramirez and Dr. Lauerman attended the AAAP in Salt Lake City in 1998. This project-funded trip was carried out after the training visit of Dr. Ramirez in the USA and Israel.

4) Drs. Levisohn and Lauerman both participated in the 12th conference of the International Organization of Mycoplasmology in Sidney, Australia, July 1998. No project funding was used for this meeting or travel. Three project-related presentations were made, one of which specifically deals with the collaborative research.

5) Drs. Icochea, Ramirez and Lauerman participated in the AAAP Meeting, New Orleans, July 1999. The oral presentations listed below were the first time that Peruvian veterinarians appeared before this prestigious forum and reported on research carried out in that country. This had a significant impact in Peru. Participation in the meeting was funded from non-project sources.

6) All the collaborating scientists participated in the XVI Latin American Poultry Congress, Lima in September 1999. This was a major international congess with more than 2000 participants from all the Latin American countries and many invited scientists from outside the region, mainly USA. The participation of Dr. Levisohn and Dr. Lauerman was paid for by the congress.

Project associated presentations and publications:

1) Ramírez, A, Icochea, E. , & Noé, N (1997). Proceedings of the Avian Mycoplasma course (in Spanish), published by the School of Veterinary Medicine, San Marcos University.

2) Ewing, ML, Lauerman, LH, Kleven, SH, Brown, MB, 1996. Evaluation of diagnostic procedures to detect Mycoplasma synoviae in commercial multiplier-breeder farms and commercial hatcheries in Florida. Avian Diseases 40: 798-806.

3) Levisohn, S., Gerchman, I., Lauerman, L.H., and Weisman, Y. The use of polymerase chain reaction (PCR) for rapid diagnosis of mycoplasma in breeder flocks. *World Poult.Sci.Assn.* 35: 1997.

4) Levisohn, S., Lublin, A., Gerchman, I., Mechani, S., Lauerman, L.H., and Weisman, Y. (1997). New insights into etiology of leg problems in turkeys. *World Vet.Poult.Assn.Proc.* XI: 92, Budapest, Hungary.

5) Lauerman, L.H. (1998). "Nucleic Acid Amplification Assays for Diagnosis of Animal Diseases", American Association of Veterinary Laboratory Diagnosticians, Turlock, CA, This is a manual which includes most of the molecular procedures used in this project.

6) Levisohn, S., Gerchman, I., Ley, D. H., Geva, Z., and Weisman, Y. (1998). Application of molecular diagnostic methods to track environmental infection of poultry breeding flocks by *M. gallisepticum*, Proc., 12th IOM Congress, Sidney, Australia, pp.75.

7) Lauerman, LH, Levisohn, S, Noe Moccetti, N, Icochia, E, Ramirez, A, van Santen, V, 1998. Evaluation of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* polymerase chain reaction assays in three countries. Proc., 12th IOM Congress, Sidney, Australia.

8) F. Goll, Jr. & L. H. Lauerman. Identification of *Mycoplasma synoviae* infected chickens and turkeys by culture, polymerase chain reaction assay and serology Proc., 12th IOM Congress, Sidney, Australia.

9) Icochea, E., Ramírez,, A., Noé, N., Levisohn, S. & Lauerman L.H. *Mycoplasma gallisepticum* and *Mycoplasma synoviae* co-infection: diagnosis by PCR in layer flocks in Peru. 136th AAAP Meeting, New Orleans, LA July 1999.

10) Ramírez, A, Z. Ramos, E. Icochea, N. Noe, S. Levisohn & L. Lauerman Detection of DNA-*Mycoplasma gallisepticum* in embryo by PCR. 136th American Association of Avian Pathologists (AAAP) Meeting, New Orleans, LA July 1999.

11) Lauerman, L. H. , 1999. DNA-based Diagnostic Methods for Avian Mycoplasmas. (Invited presentation), Proceedings, XVI Latin American Poultry Congress, Lima. p. 15-17.

12) Levisohn, S. (1999). Serological and Molecular Diagnosis of Avian Mycoplasmas, (Invited presentation), Proceedings, XVI Latin American Poultry Congress, Lima. p. 53-54.

13) Ramírez, A, Icochea, E. , Chirinos B, Noé, N. & Levisohn, S. (1999). Sensibilidad de tres pruebas serolocas en gallinas ADN-PCR positivas a Mycoplasma gallisepticum y/o Mycoplasma synoviae. Proceedings, XVI Latin American Poultry Congress, Lima, p 272.

14) Icochea I , Ramírez, A, Noé, N. y Levisohn, S. (1999). Diagnostico de sinusitis infecciosa en pavos por ADN-PCR para Mycoplasma gallisepticum, Proceedings, XVI Latin American Poultry Congress, Lima, p 273.

15) * Lockaby, SB, Hoerr, FJ, Kleven, SH, Lauerman, LH, 1999. Pathogenicity of *Mycoplasma synoviae* in chicken embryos. Avian Diseases 43: 331-337.

16) * Lockaby, SB, Hoerr, FJ, Lauerman, LH, Kleven, SH, 1998. Pathogenicity of *Mycoplasma synoviae* in broiler chickens. Vet Pathol 35(3): 178-190.

17) * Lockaby, SB, Hoerr, FJ, Lauerman, LH, Smith, BF, Samoylov, AM, Toivio-Kinnucan, M, Kleven, SH, 1999. Factors associated with the virulence of *Mycoplasma synoviae*. Avian Diseases 43: 252-261.

18) * Lockaby, SB, Hoerr, FJ, Lauerman, LH, Smith, BF, Samoylov, AM, Toivio-Kinnucan, MA, Kleven, SH, 1999. Factors associated with virulence of *Mycoplasma synoviae*. Avian Dis 43(2): 251-261.

19) Lauerman, LH, Levisohn, S, Icochia, E, Ramirez, A, Noe Moccetti, N, Li, L, 2000. Evaluation of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* polymerase chain reaction assays in various national and international laboratories. American Veterinary Medical Association, Salt Lake City, Utah.

20) Ramirez, A., E. Icochea, P. Rios, N. Noe, S. Levisohn, Correlation between antibody and DNA-PCR of *Mycoplasma synoviae* from infected pipped embryos (AAAP, Salt Lake City, 2000).

21) Levisohn, S, I. Gerchman, A. Frummer, A, and Y. Weisman, 2001. The relationship between *Mycoplasma gallisepticum* (MG) in geese and MG outbreaks in broiler-breeder flocks. Western Poultry Disease Conf, Davis, CA.

22) Ramirez, A., E. Icochea, P. Rios, N. Noe, S. Levisohn, Clearance of *Mycoplasma synoviae* from the upper respiratory tract by local treatment with antibody and tiamulin. AAAP, Boston, 2001.

* These papers utilized techniques developed in part due to project funding but were published after Dr. Lauerman finished his formal collaboration in the project.

Courses and Workshops on project-associated subjects:

1) Icochea, E., A. Ramírez & L. H. Lauerman. Short course on avian mycoplasmosis (Spanish title: Mycoplasosis Aviar, Biotecnologia Diagnostica y Control). April 1996, Lima.

2) Icochea, E. & A. Ramírez. Workshop on "Enfermedades Respiratorias en Aves", Nov. 14-15 1996, Lima, Peru.

3) Icochea, E., N. Noé & A. Ramírez. Workshop (3 sessions) on Diagnostic Biotechnology in Mycoplasmology, under the auspices of the Asociación Peruana de Microbiologia, Feb. 3-6, 1997. Lima.

4) Icochea, E., N. Noé & A. Ramírez. Workshop (2 days) on Diagnostic Biotechnology in Mycoplasmology, under the auspces of the Xth Congreso Peruano de Microbiologia y Parasitologia, April 17-18, 1997, Lambayeque (Chiclayo), Peru.

5) Icochea, E., A. Ramírez & S. Levisohn. Special session with participation of about 200 veterinarians on the subject of "Avian Mycoplasmas", under the auspices of the Peruvian Veterinary Society, Sept. 1998, Chiclayo, Peru,

6) Noé, N., "Molecular diagnosis of mycoplasmas", 1st Peruvian Biotechnology and Bioengineering Congress, Antenor Orrego University, Trujillo, Peru. Nov. 12-15, 1998.

7) Ramírez, A., "Using of biotechnology to improve the action of nutrients and animal health" 16th Panamerican Congress of Veterinary Sciences, Santa Cruz, Bolivia. November 1998

8) Icochea D'Arrigo, Eliana , Special Course on "Serological and Molecular Diagnosis in Avian Pathology" SVM / SM Univ. Post-Graduate Unit/San Marcos Univ. December 14-15, 1998.

9) Icochea D'Arrigo, Eliana "Using of biotechnology to improve the action of nutrients and animal health" 16th Panamerican Congress of Veterinary Sciences, Santa Cruz, Bolivia. November 1998

10) Icochea, Eliana , Special Course on "Serological and Molecular Diagnosis in Avian Pathology" SVM / SM Univ. Post-Graduate Unit/San Marcos Univ. December 14-15, 1998.

11) Icochea, Eliana 1999:: Guayaquil Agrarian University, Ecuador.. Master Poultry Programme . Sept. 12, 1999. Lecture: Biotechnology in the Diagnosis of Avian Mycoplasmas.

12) Icochea, Eliana 1999:: Guayaquil Agrarian University, Ecuador.. Master Poultry Programme . Sept. 12, 1999. Lecture: Biotechnology in the Diagnosis of Avian Mycoplasmas.

Graduate dissertations, supervised by project collaborators and funded in part by the project:

1) **Ramos Chávez, Z.** 1998 MG detection in ovo by PCR. (PostGraduate theses) For MS in Animal Health, School of Veterinary Medicine, San Marcos University.
2) **Ramos Zarina** 1997. (PostGraduate theses) For MS in Animal Health, School of Veterinary Medicine, San Marcos University. PCR detection of infected Mycoplasma gallisepticum and Mycoplasma synoviae in layers in Lima region farms.
3) **Grandez, Ricardo** 1998. (PostGraduate theses) For MS in Animal Health, School of Veterinary Medicine, San Marcos University. Recognition of tissue propagation of Mycoplasma gallisepticum in experimentally infectedbroilers by the immunoperoxidase assay.
4) **Gonzalez, R..** 1999. Deteccion de *Mycoplasma gallisepticum* y *Mycoplasma synoviae* mediante diagnostico microbiolologio y la prueba de PCR, en la provincia de Chincha. School of Veterinary Medicine, San Marcos Univerisity & School of Biological Sciences, Ricardo Palma Private University , Lima, Peru.
5) **Díaz Salazar, EG.** 1999. Prevalence of MG and MS in Chincha Province Commercial Layers (Undergraduate theses). School of Veterinary Medicine San Marcos University
6) **Chirinos Zegarra, Cleofé Beatriz.** 1999. (Undergraduate thesis). HI vs ELISA evaluation for Ab detectrion against MG and MS . School of Veterinary Medicine San Marcos University

7) **Saldaña Marengo, Adolfo José.** 2000. (Undergraduate theses). Efecto del fumarato hidrogenado de tiamulina sobre la persistencia del *Mycoplasma synoviae en aves de postura comercial* .School of Veterinary Medicine San Marcos University
8) **Sanchez Ingunza, Roxana**, 2001. (Undergraduate thesis). Productive performance evaluation of a laying flock vaccinated with strain MG ts-11 strain. School of Veterinary Medicine San Marcos University
9) **Ríos Patricia** , 2001. MS detection and anti-MS Ab in embryos in naturally infected chickens (PostGraduate thesis for MS in Animal Health). School of Veterinary Medicine San Marcos University.

8. Product Productivity:

The project accomplished its major operative goal: establishment of a Mycoplasma Diagnostic Unit in Peru. An important element is the project- funded Biotechnology Unit. Figure 3 shows part of the unit and equipment, together with one of the project-trained technicians. The project fostered a local research capacity, and was the impetus and stimulus for increased interaction between university scientists and field practitioners, for the benefit of each side. Figure 4 demonstrates one level of this interaction, in which bird specimens are collected in the field and brought to the lab for testing.

The improved diagnostic capacity and on-going dialogue are crucial elements in the overall goal of improving control of avian mycoplasmosis in Peru. That will be a long-term process, requiring a clearly define control strategy, introduction of guidelines, and establishment of a framework for surveillance of breeder flocks. It is also an on-going process, not only in Peru but also in Israel and even in the USA. The project has had a major role in expediting this process in Peru, and thereby serving as a model for other LDC's.

9. Future Work:

It is clear that avian mycoplasmosis will continue to be a major world-wide problem. Control strategy for mycoplasmosis is changing, in response to changes in the industry. Diagnostic techniques are constantly improving, and molecular diagnostic techniques are becoming faster and easier. Peru has made a major advance toward reaching the current level of proficiency in poultry diagnostics. The current trend in control of mycoplasmosis is the introduction of live mycoplasma vaccines in certain poultry sectors, as is already occurring in Peru. Current research in the Israeli laboratory focuses on molecular typing methods, which are the basis for epidemiological studies and are essential for monitoring use of live MG and MS vaccines. In the near future these methods will be widely applicable, and will be the basis of any future collaborative project with Peru.

The network of international connections, initiated in large part through this project, is well established. Dr. Icochea and other staff members now attend international poultry meetings and keep abreast of scientific developments. This , as evidenced in this report. He has recently moved to another veterinary university in Lima, at a high-level academic/administrative position. Only time will tell if his collaboration with the San Marcos Poultry Department will continue, and research activity in the area of avian mycoplasmosis will be sustained.

10. Literature Cited

1. Anonymous (1997). National Poultry Improvement Plan Guidelines and Regulations. Beltsville MD, U.S.D.A.

2. Bradbury, J. M., O. M. Saed, et al. (1993). "*Mycoplasma imitans* sp. nov. is related to *Mycoplasma gallisepticum* and found in birds." International Journal of Systematic Bacteriology **43**: 721-728.

3. Ganapathy, K., R. C. Jones, et al. (1998). "Pathogenicity of in vivo-passaged *Mycoplasma imitans* in turkey poults in single infection and in dual infection with rhinotracheitis virus." Avian Pathol. 27: 80-89.

4. Ganapathy, K. and J. M. Bradbury (1999). "Pathogenicity of *Mycoplasma imitans* in mixed infection with infectious bronchitis virus in chickens." Avian Pathol. 28: 229-237.

5. Kempf, I. (1998). "DNA amplification methods for diagnosis and epidemiological investigations of avian mycoplasmosis." Avian Pathol. **27**: 7-14.

6. Kempf, I., F. Gesbert, et al. (1997). "Experimental infection of ducks and chickens with *Mycoplasma imitans* 4229." Proceedings de journale avicoles 1997: 137-139.

7. Kempf, I., F. Gesbert, et al. (1994). "*Mycoplasma gallisepticum* infection in drug-treated chickens: Comparison of diagnostic methods including polymerase chain reaction." Journal of Veterinary Medicine B 41: 597-602.

8. Kuniyasu, C., Y. Yoshida, et al. (1974). "Spiramycin- and tylosin-resistant strains of *Mycoplasma gallisepticum* isolated from breeding chickens and pipped eggs." Natl.Inst.Anim.Health Q. 14: 48-53.

9. Lauerman, L. H. (1998). Nucleic Acid Amplification Assays for Diagnosis of Animal Diseases. Turlock, CA, American Association of Veterinary Laboratory Diagnosticians.

10. Levisohn, S, Glisson, JR, Kleven, SH, 1985. In ovo pathogenicity of *Mycoplasma gallisepticum* strains in the presence and absence of maternal antibody. Avian Diseases 29: 188-197.

11. Levisohn, S., H. C. Hyman, et al. (1989). "The use of a specific DNA probe for detection of *Mycoplasma gallisepticum* in field outbreaks." Avian Pathol. 18: 535-541.

12. Levisohn, S. (1994). Chemotherapy of avian mycoplasmosis: In *vitro, in vivo* and clinical reality. 9th European Poultry Conference, Glasgow, Scotland.

13. Levisohn, S. and S. H. Kleven (2000). Avian mycoplasmosis (*Mycoplasma gallisepticum*). Diseases of Poultry: world trade and public health implications. C. W. Beard and S. McNulty. Paris, France, Office international des epizooties. 19: 425-442.

14. Levisohn, S., I. Gerchman, et al. (2001). The relationship between *Mycoplasma gallisepticum* (MG) in geese and MG outbreaks in broiler-breeder flocks. Western Poultry Disease Conf, Davis, CA.

15. Ley, D. H. and H. W. Yoder (1997). *Mycoplasma gallisepticum* Infection. Diseases of Poultry. B. W. Calnek, H. J. Barnes, C. Beard, L. R. McDougald and Y. M. Saif. London, Mosby-Wolfe: 194-207.

16. Marois, C, Oufour-Gesbert, F, Kempf, I, 2000. Detection of *Mycoplasma synoviae* in poultry environment samples by culture and polymerase chain reaction. Vet Microbiol 73(4): 311-318.