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# Isolation and Progeny Transmission of Non-Temperature-Sensitive MS-H Vaccine Strains of *Mycoplasma synoviae* from Temperature-Sensitive MS-H-Vaccinated Laying Breeder Hens

**Victor M. Petrone-Garcia[1*], Raquel López-Arellano[2], Inkar Castellanos-Huerta[3], Saeed El-Ashram[4], Ebtesam Al-Olayan[5], Danielle Graham[3], Guillermo Tellez-Isaias[3]**

[1]Departamento de Ciencias Pecuarias, Universidad Nacional Autónoma de México (UNAM), Cuautitlan Izcalli, Estado de México, México
[2]Laboratorio No 5: LEDEFAR, Unidad de Investigacion Multidisciplinaria, Facultad de Estudios Superi-ores Cuautitlan, UNAM, Cuautitlan Izcalli, Estado de México, México
[3]Department of Poultry Science, University of Arkansas, Fayetteville, USA
[4]Department of Zoology, Faculty of Science, Kafrelsheikh University, Kafr El-Sheikh, Egypt
[5]Department of Zoology, College of Science, King Saud University, Riyadh, Saudi Arabia
Email: *vmpetrone@hotmail.com, lopezar@unam.mx, castellanos.inkar@gmail.com, saeed_elashram@yahoo.com, eolayan@ksu.edu.sa, bmahaffe@uark.edu, gtellez@uark.edu

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## Abstract

This pilot study reports the vertical transmission and reverse thermosensitivity of the MS-H vaccine strain of *Mycoplasma synoviae* (MS) by RAPD in commercial breeders and their progeny. At two weeks of age, breeders were vaccinated with the ts$^+$ MS-H strain. At 9 weeks of age, an outbreak of infectious synovitis (IS) was detected in the progeny. Tracheal swab samples were collected from breeders at 24, 39, 48, and 70 weeks of age. At 9 weeks, pullets swab from the elbow joints were collected. RAPD was performed on the isolates at 39.5˚C, and the same ts$^-$ MS-H strains were identified in the breeder hens and their progeny. Tracheal swabs from breeder hens were negative to MS isolation at 37˚C and 39.5˚C at 24- and 39-weeks. MS isolation was recovered from tracheal swabs from 9/10 and 10/10 breeders at 48- and 70-week. At 9 weeks of age in the progeny, MS was isolated from tracheal swabs of 10/10 from non-IS pullets. MS was isolated from 9/10 joints samples. The isolates from breeder hens and their progeny showed non-significant differences in five antimycoplasmic MIC100 values; otherwise, enrofloxacin presented a significant difference in MIC100 value ($p < 0.05$). This investigation demonstrated the reversal of the thermosensitivity, pathogenicity, and vertic-

al transmission of the MS-H strain. Consequently, it is crucial to contemplate the danger of reversing pathogenicity and transmission to progeny when applying the MS-H vaccine strain.

## Keywords

Vertical Transmission, Thermosensitivity, *Mycoplasma synoviae*, RAPD, Vax-Safe®

## 1. Introduction

*Mycoplasma synoviae* (MS) is the main *Mycoplasma* species in Mexican poultry production [1]. The route of MS infection may play a significant part in the resulting disease. Natural infection may occur vertically in ovo or horizontally by direct contact or airborne spread [2]. MS isolated from air sac lesions are more apt to cause airsacculitis, whereas those isolated from synovia are more apt to produce synovitis [3]. Vertical transmission plays a major role in spread of MS in chickens; however, several flocks hatched from infected breeders may remain free of infection (Vardaman). Vaccination and antibiotic treatments principally focus on leghorn breeder hens, have been used to prevent the vertical transmission of *Mycoplasma synoviae* [2]. In several countries, the live attenuated *Mycoplasma synoviae* strain MS-H, a temperature-sensitive ($ts^+$) strain at <39.5˚C is used as a vaccination method to reduce virulent MS infection in commercial poultry. The vaccine is produced via chemical mutagenesis of the Australian field strain 86079/7NS [4]. Vaccination with the $ts^+$ MS-H strain has the purpose of colonizing the extrapulmonary respiratory tract without causing systemic infection and transovarial transmission to the progeny. Nevertheless, the reversion from $ts^+$ to temperature no-sensitive ($ts^-$) (≥39.5˚C insulation) of live attenuated *Mycoplasma* spp. vaccines has been reported to revert virulence and vertical transmission to the progeny, produce infection of the air sacs of infected hens, and the $ts^-$ isolates that lacked the $ts^+$ characteristic of the original vaccine strain generated significantly higher tracheal mucosal thicknesses than MS-H-inoculated birds [5]. Moreover, recent studies have also shown reversion to virulence and vertical transmission of the ts-11, a live *Mycoplasma gallisepticum* vaccine [6] [7]. While the $ts^-$ MS-H vaccine has received wide use in many countries over the past twenty five years, registration in the USA, the largest poultry producer country in the world has not been granted [2]. The authors of this paper asked 2022 the U.S. authorities why the MS-H vaccine has not been granted registration in the U.S. The authorities replied that the information is the company's property that carried out the registration process in the U.S.A. The information related to this request depends on the final decision of the company to share sensitive information. However, the company still needs to send us ungranted registration information. Another alternative for the control of mycoplasmosis is antibiotic metaphylaxis. The antibiotics most widely used

against *Mycoplasma* spp. are doxycycline, enrofloxacin, erythromycin, lincomycin, tiamulin, and tylosin [8] [9]. However, MS has been able to persist despite fluoroquinolone treatments in hens after two enrofloxacin treatments [10].

The random amplification of polymorphic DNA (RAPD) is based on the analysis of bacterial samples of total genomic DNA subjected to PCR using short oligonucleotides of random sequences. This amplification protocol differs from standard PCR conditions because only a single random primer is used, and prior knowledge of the genome under analysis is not required [11]. This strategy provides an inherent advantage to de novo assays: not requiring prior sequencing studies and previous primers designed. Using a short (10-bp) primer, there is a high probability that the genome contains several amplification sites close to each other in an inverted orientation. The technique searches genomic material for small inverted sample repeats and amplifies intermediate segments of DNA of various lengths. The amplification product will depend on the template/primer combination and is reproducible for any combination. Amplification products are observed directly on agarose gels; Polymorphisms are considered dominant genetic markers inherited in a Mendelian genetic. The RAPD recognition technique has been demonstrated to be efficient, as well as especially effective for the epidemiological research of *Mycoplasma gallisepticum* (MG) strains and for the classification and distinction of vaccine strains and field isolates [1] [6] [12] [13] [14] [15]. However, the efficacy of RAPD in differentiating vaccine and field strains of MS has not been demonstrated. Marois *et al.* demonstrated that RAPD has a discriminatory indicator for MS from MG which is more significant than 0.95; therefore, RAPD was selected for the present work [16].

Recently, our laboratories published a study showing the reverse of thermosensitivity of the $ts^+$ MS-H strain [1]. However, as far as we are aware, there are no evidence on the reverse pathogenicity of the $ts^+$ MS-H strain in poultry production, and the transmission to the progeny of hens vaccinated with $ts^+$ MS-H, as has been previously reported with $ts^+$ live attenuated MG vaccines [6] [7]. Therefore, the present study aimed to conduct a pilot study to lay the groundwork for evaluating the utility of the random amplification of polymorphic DNA (RAPD) method to analyze vertical transmission and possible reversal of thermosensitivity of the MS-H vaccine strain of *Mycoplasma synoviae* and evaluate the minimum inhibitory concentrations (MIC100) of the isolates (at 39.5˚C) in Hy-line® commercial breeder hens and progeny from the same parental batch.

## 2. Materials and Methods

### 2.1. Animal Sources

Hy-line® breeders and their progeny were obtained from two farms located in distant counties (50 km) in the Mexican state of Jalisco from the same integrated company. At two weeks of age, breeder hens were vaccinated with the live attenuated strain $ts^+$ MS-H (Vax-safe® MS, Bioproperties® Ringwood VIC 3134, Autralia). At 9 weeks of age, an outbreak of IS was detected in the unvaccinated fe-

male progeny. Pullets were then treated with 20 mg·mL$^{-1}$ enrofloxacin (Baytril®, Elanco, Greenfield, IN, USA. The technical data sheet indicates a dose of 10 mg·kg$^{-1}$ of body weight per day) in the drinking water for 5 consecutive days [8]. This treatment was administered twice a day (morning and evening) to control the IS.

### 2.2. Sample Collection

Tracheal swab samples (n = 40) were collected from breeder hens farm were collected at 24, 39, 48, and 70 weeks of age. In the pullet farm, at 9 weeks of age, tracheal swab samples (n = 10) were collected pullets with no clinical signs of synovitis. Furthermore, pullets showing clinical signs of arthritis were euthanized by cervical dislocation to collect swab samples from the trachea (n = 10) and elbow joints with the presence of serofibrinous fluid (n = 10).

### 2.3. Bacterial Isolation

Swab samples were incubated at 39.5˚C for 21 days [17]. The cultures were carried out according to the methodologies described by Petrone-Garcia *et al.* [1]. To serve as controls, original strains of the MS-H vaccine and the ts$^{+}$ TS-11 MG strain (TS-11®, Bioproperties® Ringwood VIC 3134, Autralia) were incubated at 33˚C. The ts$^{-}$ F MG strain (F VAX MG®, MSD Animal Health, Mexico City, Mexico) was incubated at 37˚C and 39.5˚C.

### 2.4. RAPD Identification of *Mycoplasma synoviae* Isolations

The DNA of cloned MS strains was characterized by RAPD, utilizing the method and primers proposed by Geary *et al.* [18]. The DNAbp of the isolated strains were matched to the DNAbp of the MS-H (33˚C), F (37˚C and 39.5˚C), and TS-11 (33˚C) reference strains.

### 2.5. Minimum Inhibitory Concentrations

*Mycoplasma synoviae* isolated from swab samples in each group respectively, were subjected to MIC100. MIC100 values were described as the lowest concentration of the antibiotic at which 100% of the isolates were inhibited. The antimycoplasmics used were doxycycline, enrofloxacin, erythromycin thiocyanate, lincomycin hydrochloride, tiamulin hydrogen fumarate, and tylosin tartrate (Merck, Kenilworth, NJ, USA). The MIC100 values were estimated in correspondence to the final dilution of the base molecule. The MIC100 was analyzed according to the technique published by Hannan [19] and Gautier-Bouchardon [20] and appropriated by Petrone-Garcia *et al.* [1]. Two-fold dilutions of the antibiotic were made, fluctuating from 5 to 0.01 μg·mL$^{-1}$.

#### Statistical Analysis

For MIC100, the data are expressed as the mean and median. The minimum number of samples per group of variables was ten. The hypothesis of normal distribution was verified with the Shapiro-Wilk test and homoscedasticity with

Levene's test; however, the two statistical conditions were not established. Subsequently, the Kruskal-Wallis non-parametric tests were applied and, afterward, the Mann-Whitney U test was carried out, in order to compare between pairs of groups. The statistical significance was set at $p < 0.05$.

## 3. Results

### 3.1. Bacterial Isolation

Tracheal swabs from breeder hens were negative to MS isolation at both temperatures (37˚C and 39.5˚C) at 24- and 39-week-old breeder hens. However, MS isolation was recovered from tracheal swabs from 9/10 (90%) and 10/10 (100%) breeder hens at 48- and 70-week-old. MS was isolated from tracheal swabs of 10/10 (100%) from non-synovitis pullets at nine weeks of age in the progeny. MS was isolated from 9/10 (90%) trachea and elbow joint samples in pullets with the presence of serofibrinous fluid.

### 3.2. Molecular Identification (RAPD) of *Mycoplasma synoviae*

All the isolates obtained from breeder hens and their progeny (with or without IS) exhibited identical DNA banding patterns (DNAbp) corresponding to those of the MS-H strain (Figure 1).

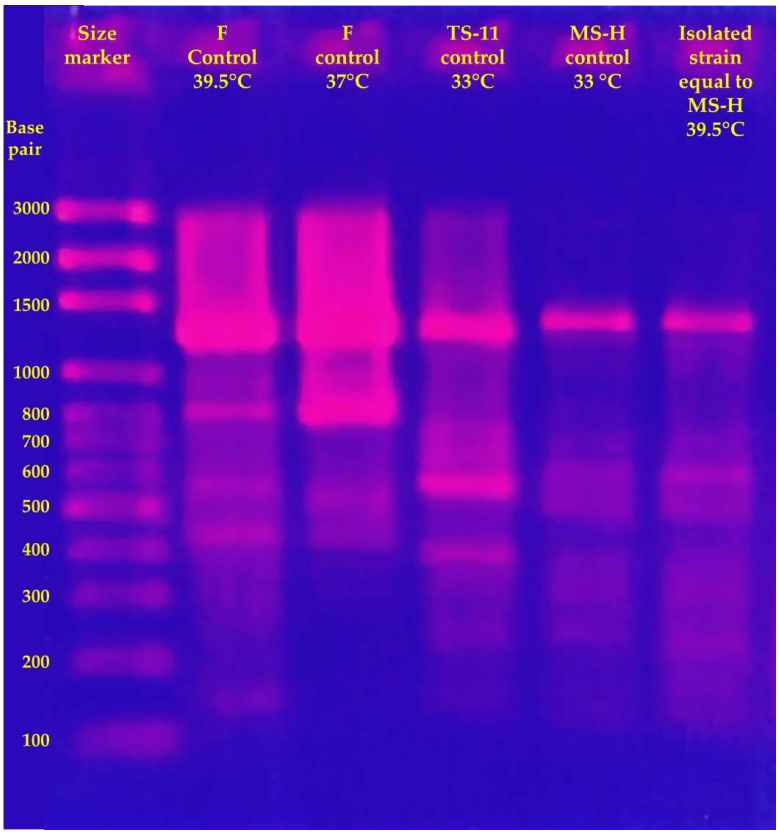


Figure 1. RAPD patterns showing *Mycoplasma synoviae* isolate from the IS progeny group (MS-H isolate 39.5˚C). 39.5˚C isolate strain presenting DNAbp matching those of the MS-H vaccine strain.

### 3.3. Minimum Inhibitory Concentrations

No MIC100 differences ($p > 0.05$) were observed between breeders and their progeny for doxycycline, erythromycin, lincomycin, or tylosin; however, different MIC100 values ($p < 0.05$) were observed between breeders and their progeny for enrofloxacin, and between breeders and progeny with clinical signs or progeny with non-clinical signs. Progeny with no presence of synovitis showed a significant reduction in MIC to Tiamulin compared with breeder hens (Table 1).

## 4. Discussion

In the present field report, isolation, and identification of the MS-H strains from tracheal samples of breeder hens at 48 and 72 weeks was demonstrated. Interestingly, the strains isolated from tracheal swabs grew at 37˚C and 39.5˚C. The $ts^-$ MS-H strains isolated at 39.5˚C showed DNAbp equal to those of the $ts^+$ MS-H vaccine strain. Moreover, the $ts^-$ MS-H strain, with similar DNAbp as $ts^+$ MS-H vaccine strain, was also isolated in progeny chickens in pullets showing clinical signs of synovitis or apparently healthy pullets. The laying breeder hens in the present study were positive for the MS-H strain, both by isolation and RAPD, until 48 and 70 weeks of age, indicating that the $ts^+$ MS-H strain was slow to revert thermosensitivity. However, when the strain was transmitted to progeny, it produced synovitis at nine weeks of age. These findings suggest failures in vaccine immunity, mixed infection between the vaccine strain and the field strain, or the possibility that the $ts^+$ strain can reverse its thermosensitivity at 39.5˚C, virulence, and replicate in laying breeder hens. In addition, the $ts^-$ strain can be vertically transmitted to offspring from the same parent flock.

In a study published by Noormohammadi *et al.*, 2003, 9/50 isolates from flocks vaccinated with the $ts^+$ MS-H strain were found to have lost the $ts^+$ phenotype of the original vaccine strain. In 2/9 $ts^-$ MS clones were recovered from air sacs and 3/9 isolates produced increased tracheal mucosal thicknesses compared to MS-H inoculated birds, and 1/9 induced increased tracheal mucosal thicknesses [5].

**Table 1.** Minimum inhibitory concentration100 (MIC100) of *Mycoplasma synoviae* isolates (39.5˚C) from laying breeder hens and their progeny.

| Groups | Doxycycline | Enrofloxacin | Erythromycin | Lincomycin | Tiamulin | Tylosin |
|---|---|---|---|---|---|---|
| Breeder hens (n = 20) | 0.734 (0.625) | 0.640[a] (0.625) | ≥5.000 (5.000) | 2.469 (2.500) | 1.281[b] (1.250) | 0.049 (0.039) |
| Non-IS[†] progeny (n = 10) | 0.844 (0.625) | ≥5.000[b] (5.000) | ≥5.000 (5.000) | 1.500 (1.250) | 0.390[a] (0.312) | 0.047 (0.039) |
| IS progeny (n = 10) | 1.000 (1.250) | ≥5.000[b] (5.000) | ≥5.000 (5.000) | 2.375 (2.500) | 0.750[a,b] (0.625) | 0.045 (0.039) |

Data expressed as mean (median). [†]IS = infectious synovitis. Dissimilar letters within the same column show significant differences ($p < 0.05$).

The reverse of thermosensitivity has been also demonstrated with other commercial live attenuated *Mycoplasma* vaccines. In a study published by El Gazzar *et al.*, 2011 from commercial broiler flocks in northeastern Georgia, USA that descended from the same parent flock were found to be *Mycoplasma gallisepticum* (MG) positive using serology, culture, and PCR, indicating reversion to virulence and vertical transmission of the ts-11 live MG vaccine [7]. Other scientists have also provided conclusive evidence of transovarian transmission and reverse virulence of the isolate genotyped as MG ts-11 [6].

In the present, no differences were observed in MIC100s between laying breeder hens and progeny for doxycycline, erythromycin, lincomycin, and tylosin. However, a significant increase in MIC100 for enrofloxacin was observed in the progeny, following the standard therapeutic treatment of the poultry company. The ability of MS to persist in spite of fluoroquinolone treatments has been previously demonstrated by Le Carrou *et al.*, 2006 who found a significant increase in the resistance level to enrofloxacin of five reisolated *Mycoplasma* clones, which was observed after the second treatment [10]. Furthermore, we found that the antimycoplasmic agent with the highest sensitivity for MS was tylosin, and this result is in agreement with a previous study [1]. Remarkably, in a study published by Pakpinyo and Sasipreeyajan [13], the molecular characterization and determination of MIC tested on MG isolated from commercial chickens had similar RAPD patterns and properties of antimicrobial resistance, providing useful information to plan for prophylactic and therapeutic impacts on the poultry industry.

## 5. Conclusion

The results of this field report suggest that vaccination failed or that the strain of the live attenuated ts$^+$ MS-H vaccine can revert its temperature sensitivity to perform vertical transmission to the progeny, recover virulence, and induce synovitis. The RAPD technique could easily differentiate between the different avian *Mycoplasma* species and differentiate between MG vaccine and field strains. However, for MS strains, there are no reports showing differences between strains. The underreporting may be since the RAPD method for interpreting MS isolates does not distinguish between genetic variation among MS strains during an outbreak and the presence of different but closely related strains. Hence, it is important to contemplate the potential danger when using ts$^+$ MS-H is a reversion of ts$^+$ with a possible reversion of pathogenicity to the original Australian field strain 86079/7NS and vertical transmission to progeny when using the ts$^+$ MS-H vaccine strain or other life ts$^+$ vaccines against *Mycoplasma* spp. This work should be taken as a pilot study for further studies confirming the findings of this field report under experimental and laboratory conditions and ruling out or confirming the use of the RAPD method to differentiate MS strains.

## Authors' Contributions

Conceptualization, V.M.P-G.; methodology, V.M.P.-G.; software, V.M.P.-G.; va-

lidation, G.T.-I., R.L.-A, S. E.-A., E.O.; formal analysis, G.T.-I.; investigation, V.M.P.-G.; resources, V.M.P.-G. data curation, I.C.-H. and D.G.; writing—original draft preparation, V.M.P-G.; writing—review and editing, V.M.P-G, G.T.-I., R.L.-A., and F.A.-H; visualization, V.M.P.-G; project administration, G.T.-I., and R.L.-A.; funding acquisition, G.T.-I., R.L.-A., S. E.-A., E.O. and V.M.P.-G. All authors have read and agreed to the published version of the manuscript.

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## Conflicts of Interest

The authors declare no conflict of interest.

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