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Case report

# Monitoring for *Mycoplasma hyopneumoniae* before and after a partial depopulation program using a typing scheme based on the polyserine repeat motif of *p146*

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## Summary

*Mycoplasma hyopneumoniae* diversity was determined using a molecular typing method based on the polyserine repeat motif within the *p146* gene. Three related Argentinian farms (A, B, and C) were investigated. To obtain a population free of enzootic pneumonia on Farm C, a partial depopulation program had been carried out first on Farm A and then on Farm B. Finally, Farm C was populated with early-weaned piglets from Farm B. To evaluate the success of the partial depopulation program, the farms were monitored for clinical signs and by serological testing, lung examination at slaughter, and nested polymerase chain reaction (nPCR). It was concluded that they were free of enzootic pneumonia, but *M hyopneumoniae* remained despite the eradication measures applied. An outbreak of enzootic pneumonia in Farm C triggered an investigation of *M hyopneumoniae* genetic diversity in these farms. For this purpose, all DNA samples obtained from PCR-positive nasal swabs were further characterized using another nPCR designed for *M hyopneumoniae* typing. Several *M hyopneumoniae* types were identified in these farms, but one strain seemed to be present before and after the application of the partial depopulation program. Unambiguous discrimination of *M hyopneumoniae* would require analysis of other genomic regions.

**Keywords**: swine, *Mycoplasma hyopneumoniae*, typing, persistence, polymerase chain reaction.

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**Resumen - Monitoreo del *Mycoplasma hyopneumoniae* antes y después de un programa de despoblación parcial utilizando tipificación basado en el esquema de repetición de la poliserina *p146***

Se determinó la diversidad del *Mycoplasma hyopneumoniae* utilizando un método de tipificación molecular basado en el esquema de repetición de la poliserina dentro el gen *p146*. Se investigaron tres granjas argentinas relacionadas (A, B, y C). Para obtener una población libre de neumonía enzoótica en la Granja C, se llevó a cabo un programa de despoblación parcial, primero en la Granja A y luego en la Granja B. Finalmente, se pobló la Granja C con lechones de destete temprano de la Granja B. Para evaluar el éxito del programa de despoblación parcial, las granjas se monitorearon en busca de signos clínicos y por medio de pruebas serológicas, examen de pulmones en el matadero, y por medio de la prueba de reacción en cadena de la polimerasa anidada (nPCR). Se concluyó que estaban libres de neumonía enzoótica, pero que el *M hyopneumoniae* permaneció a pesar de las medidas de erradicación aplicadas. Un brote de neumonía enzoótica en la Granja C desencadenó una investigación de la diversidad genética del *M hyopneumoniae* en estas granjas. Para este propósito, todas las muestras de DNA obtenidas de hisopos nasales positivos fueron caracterizados más a fondo utilizando otro nPCR designado para la tipificación del *M hyopneumoniae*. Se identificaron varios tipos de *M hyopneumoniae* en estas granjas, una cepa pareció estar presente antes y después de la aplicación del programa de despoblación parcial. La discriminación definitiva del *M hyopneumoniae* requeriría del análisis de otras regiones genómicas.

**Résumé - Monitorage de *Mycoplasma hyopneumoniae* avant et après un programme de dépopulation partielle utilisant un schéma de typage basé sur le motif répété de la polysérine de *p146***

La diversité de *Mycoplasma hyopneumoniae* a été déterminée au moyen d'une méthode de typage moléculaire basée sur le motif répété de la polysérine au sein du gène *p146*. Trois fermes argentaises reliées (A, B, et C) ont été étudiées. Afin d'obtenir une population exempte de pneumonie enzootique sur la Ferme C, un programme de dépopulation partielle a été mené en premier lieu sur la Ferme A et par la suite sur la Ferme B. Finalement, la Ferme C a été peuplée avec des porcelets sevrés hâtivement provenant de la Ferme B. Afin d'évaluer le succès du programme de dépopulation partielle, les animaux sur les fermes ont été surveillées pour la présence de signes cliniques ainsi qu'au moyen de tests sérologiques, l'examen des poumons à l'abattoir, et par réaction

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nichée d'amplification en chaîne par la polymérase (nPCR). Il a été conclu qu'ils étaient exempts de pneumonie enzootique, mais que *M hyopneumoniae* persistait malgré les mesures d'éradication appliquées. Une poussé de cas de pneumonie enzootique sur la Ferme C a déclenché une étude sur la diversité génétique de *M hyopneumoniae* dans ces fermes. À cette fin, tous les échantillons d'ADN obtenus des écouvillons nasaux positifs par PCR ont été caractérisés plus à fond en utilisant une autre épreuve nPCR élaborée pour le typage de *M hyopneumoniae*. Plusieurs types de *M hyopneumoniae* furent identifiés sur ces fermes, mais une souche semblait être présente avant et après la mise en place du programme de dépopulation partielle. Une discrimination claire de *M hyopneumoniae* nécessiterait une analyse génomique de d'autres régions du génome.

*Mycoplasma hyopneumoniae* is the primary agent involved in porcine enzootic pneumonia. Infections with *M hyopneumoniae* are highly prevalent in almost all swine-producing areas, causing significant economic losses to the pig industry worldwide.[1] Control of *M hyopneumoniae* infections can be accomplished in several ways, mainly by optimization of management practices and the use of antimicrobials and vaccines.[2]

Partial depopulation programs have been used to eradicate *M hyopneumoniae* in herds of several sizes,[3-5] with an estimated success rate of approximately 80% to 90%.[6] To monitor the success of these programs, diagnostic strategies such as clinical examination, serological testing of herds, and inspection of lung lesions at slaughter have been used. Furthermore, molecular techniques such as polymerase chain reaction (PCR) are very useful due to their high sensitivity and specificity. To maintain enzootic pneumonia-free status, herds should be monitored by diagnostic techniques suitable for *M hyopneumoniae* typing to help identify the source of new *M hyopneumoniae* infections or re-infections, and so enforce and correct control measures to warrant success on control or eradication of the disease.

Genetic typing of *M hyopneumoniae* based on the region of the *p146* gene that codes for a serine repeat motif has been applied to characterize and discriminate among *M hyopneumoniae* strains in several studies,[7-9] demonstrating a high variability among different herds and geographical locations. This method was also useful for investigation of a new *M hyopneumoniae* infection in a previously negative herd [10] and to support the hypothesis that long-distance airborne transport of the agent can occur.[11,12] However, this approach has not been applied to study the efficacy of an eradication program, providing information about the genetic types of *M hyopneumoniae* present.

This case report describes one farrow-to-finish and two commercial multiple-site farms in Argentina that became free of enzootic pneumonia after a partial depopulation program, but *M hyopneumoniae* was still detectable by nested PCR (nPCR). An outbreak of enzootic pneumonia in one of these farms prompted us to investigate *M hyopneumoniae* genetic diversity before and after application of the partial depopulation program. *Mycoplasma hyopneumoniae* typing was based on the polyserine repeat motif encoded by the *p146* gene, and the present study reports the results obtained.

## Application of partial depopulation programs and monitoring of enzootic pneumonia-free offspring: historical data

The study was performed according to the international guidelines of the Council for International Organizations of Medical Sciences (CIOMS).

Three related farms (A, B, and C) belonging to the same company were investigated. All farms were located in the main swine-producing area of Argentina. Farm A was a one-site, 390-sow farrow-to-finish herd, supplier of replacement animals to Farm B. Farm B was a 4100-sow commercial three-site farm. Farm C, at the time of the study, had just been built, with new facilities beginning to be populated with animals from Farm B. Farms A and B were less than 8 km from each other, and Farm C was approximately 460 km from either Farm A or Farm B. To obtain an enzootic pneumonia-free population on Farm C, the company decided to carry out a partial depopulation program first on Farm A and then on Farm B.

Briefly, animals younger than 10 months were removed, and only the breeding animals remained (sows, gilts, and boars), which were hyperimmunized and medicated with in-feed antibiotics (tiamulin, 100 g per tonne [Dynamutilin 10% Premix; Novartis Animal Health, Kundl, Tirol, Austria] and chlortetracycline, 300 g per tonne) by using pulses of 15 days duration for a 4-month period. Mating and breeding processes were interrupted and the facilities were cleaned and disinfected.

To assess the success of the program, three groups of sows (a total of 42 sows in Farm A and 82 sows in Farm B, pre- and post-farrowing) and their offspring at 2, 8 to 10, 15, and 21 weeks of age (a total of 112 pigs in Farm A and 163 pigs in Farm B) were monitored by observation of clinical signs, serological testing (HerdChek *M. hyopneumoniae* Antibody ELISA Test Kit; Idexx Laboratories, Inc, Westbrook, Maine), and testing of nasal swabs by nPCR for detection of *M hyopneumoniae*.[13] At slaughter, gross lung lesions suggestive of enzootic pneumonia were assessed, and suspect samples were examined by histopathological analysis.[14]

To populate Farm C, piglets from Farm B were early weaned (7 to 9 days of age), treated once with an injectable antibiotic (tulathromycin, 2.5 mg per kg), and then transferred to the new facilities on Farm C twice a week for a period of 10 weeks. Ten groups (one group per week) of 100 pigs were monitored by observation of clinical signs, ELISA testing, and inspection of lung lesions as described. In addition, nasal swabs from the first three groups were monitored at 21 weeks of age by nPCR for detection of *M hyopneumoniae*.[13]

Despite the use of control strategies, it was concluded that, although the disease (enzootic pneumonia) was eradicated, *M hyopneumoniae* remained in the three herds. This conclusion was supported by detection of *M hyopneumoniae* by nPCR, concurrent with the absence of clinical signs, a low percentage of seropositive animals (which had low ELISA titers), and lung lesions observed histologically that could have been caused by other pathogens.[14]

## *Mycoplasma hyopneumoniae* typing

*Mycoplasma hyopneumoniae* typing was performed to determine *M hyopneumoniae* diversity before and after the partial depopulation program on farms A and B, and to study the initial population on Farm C. The criterion to define before ("Before") and after ("After") the partial depopulation

program was the following: we assumed that Before could be represented by the sow population, since they were never removed from the facilities, and After by the offspring born after the hiatus on farrowing. The initial population on Farm C was considered a post-eradication population.

All DNA samples selected for typing had tested positive for *M hyopneumoniae* in a previously reported study[14] using the nPCR protocol described by Calsamiglia et al.[13] All DNA samples had been extracted from nasal swabs using a commercial kit (DNAzol; Invitrogen, Carlsbad, California) and were stored at -20°C. According to our previous experience, the genetic material obtained from nasal swabs could not be amplified by a standard PCR, and therefore the samples (n = 189) were analyzed by the nPCR developed by Tamiozzo et al,[9] targeting the region of the *p146* gene that codes for a serine repeat motif. This protocol comprises a first reaction performed with the primers described by Tamiozzo et al[9] and a second reaction using 2 µL of the first reaction product and the conditions and primers described by Mayor et al.[7]

Twenty-five samples rendered a product with the nPCR used for *M hyopneumoniae* typing. Polymerase chain reaction products were purified (QIAquick PCR Purification Kit; Qiagen, Foster City, California), quantified, and sequenced (ABI 3130*xl*; Applied Biosystems, Foster City, California) with the primers proposed by Mayor et al.[7] The number of serine repeats (which were encoded by the codons TCT, TCA, and TCC) was determined by viewing the sequences with the Chromas 2.32 software (Technelysium Pty Ltd, Brisbane, Australia). Typing results are shown in Table 1. On Farm A, only one strain of *M hyopneumoniae* was identified, with a series of 21 serine repeats, both in six sows (Before) and one 21- to 22-week-old pig (After). On Farm B, three different *M hyopneumoniae* strains were detected: one with 16 serine repeats from a sow (Before), another with 21 repeats from five of the offspring (After; 2-week-old piglets), and the third one, with 14 repeats, detected in a sow (Before) and a 21- to 22-week-old pig (After). Among the population of Farm C, only one type, with 14 repeats, was identified in all animals of the initial population that had been nPCR-positive (10 pigs).

## Discussion

The fact that *M hyopneumoniae* remained on farms A and B after the application of a partial depopulation program and was also present among the initial population of Farm C has been previously discussed.[14] In that report, it was concluded that the clinical-pathological entity was eradicated, but *M hyopneumoniae* remained, as it was detected by nPCR although clinical signs (cough) were absent, lung lesions were not specific, and a low percentage of seropositive 21- to 22-week-old pigs was observed. The present results support this conclusion, as some of the genetic types of *M hyopneumoniae* identified were detected both before and after the partial depopulation program.

Not all the DNA samples from nasal swabs that had tested positive for *M hyopneumoniae* could be amplified by the nPCR used in this study. This could be due either to a greater sensitivity of the nPCR designed for *M hyopneumoniae* detection[13] than the nPCR designed for typing *M hyopneumoniae* strains,[9] to variability in the primer binding sites, to DNA degradation (because repeated freezing and thawing of DNA samples may shear the DNA), or to false-positive results for *M hyopneumoniae* in the previous analysis of the samples, although precautionary measures were taken to prevent cross contamination. However, this is not the focus of this report.

Even with a limited number of positive samples, it was possible to detect *M hyopneumoniae* diversity before and after application of the partial depopulation programs. On Farm A, only one strain of *M hyopneumoniae* could be identified, both before and after application of the partial depopulation program. This was a farrow-to-finish herd, and surely the fact that it was a one-site herd favored *M hyopneumoniae* dissemination in spite of the application of the partial depopulation program, due to intermittent elimination of the agent and progressive spread of *M hyopneumoniae* in farrow-to-finish herds[15] compared to multiple-site systems.

Nevertheless, on Farm B, three different *M hyopneumoniae* strains could be detected, one of them present both before and after application of eradication measures. The existence of more than one strain within a herd has been previously reported by Vranckx et al,[16] who found differences in diversity and persistence of *M hyopneumoniae* strains among herds, probably related in some cases to management practices characteristic of each farm. An *M hyopneumoniae* type with 21 serine repeats was found on farms A and B, while a type with 14 serine repeats was found on farms B and C. It has been well documented

**Table 1:** Genetic typing of *Mycoplasma hyopneumoniae* DNA extracted from nasal swabs of sows and their offspring on three Argentinian swine farms*

| | Farm A | | Farm B | | Farm C |
|---|---|---|---|---|---|
| **Animals tested** | **Sows Before** | **Offspring After** | **Sows Before** | **Offspring After** | **Initial population** |
| No. of serines (no. of samples) | 21 (6) | 21 (1)† | 14 (1); 16 (1) | 14 (1)†; 21 (5)‡ | 14 (10)† |

* Two related Argentinian swine farms (A and B) underwent a partial depopulation program to eradicate *M hyopneumoniae*. Farm C was a new site populated with early-weaned offspring from Farm B. Typing was performed to assess the genetic diversity between Before (sows tested) and After (offspring tested) the partial depopulation program on farms A and B, and to study the initial population on Farm C. The number of serines encoded in the repeat motif of the *p146* gene in DNA from nasal swabs samples was determined by a nested polymerase chain reaction using as outer primers those described by Tamiozzo et al[9] and as inner primers those described by Mayor et al.[7] Sequencing was performed using this last primer pair.

† 21- to 22-week-old pigs.

‡ 2-week-old piglets.

that *M hyopneumoniae* can be easily spread among farms[17,18] and that long-distance airborne transmission of an *M hyopneumoniae* strain can occur as far as 9.2 km.[12] In this case, farms A and B were approximately 7.6 km from each other, but due to the operational proximity between farms and animal flow before application of the partial depopulation program, it is possible that entry of carrier pigs was responsible for transmission of an *M hyopneumoniae* strain from Farm A to Farm B. However, other routes of transmission, such as fomites[19] or personnel,[20] could have also played an important role.

Farm C was approximately 460 km from the others. In this case, trucks that transferred the initial population, personnel, or carrier pigs might be responsible for transport of the same *M hyopneumoniae* strain from Farm B to Farm C. The source of infection for Farm C might have been explained if further epidemiological testing had been performed.

More discriminatory molecular tools, simultaneously targeting different genomic regions, such as multiple-locus variable number tandem repeat (VNTR) analysis, have been reported as useful to determine *M hyopneumoniae* genetic diversity in clinical samples without prior cultivation[21] and have been also been applied to study the dynamics of infection.[16] In these cases, standard PCRs were performed using DNA extracted from bronchoalveolar lavage fluid and tracheal swabs. In the present study, the main limitation to analyzing other regions of the genome was the sensitivity of the PCRs, since in our experience it is difficult to detect *M hyopneumoniae* from nasal-swab samples unless an nPCR is performed. Therefore, to determine *M hyopneumoniae* genetic diversity in clinical samples without killing animals or performing invasive sampling, development of nPCRs targeting different VNTR loci is needed for the study of nasal-swab samples.

Before, during, and after application of control or eradication programs, identification of the source of *M hyopneumoniae* infection, as well as other pathogens, is crucial for the adoption, implementation, development, and surveillance of control strategies to warrant disease-free status. This report shows that the nPCR targeting the polyserine repeat motif of the *p146* gene was useful for typing *M hyopneumoniae,* a fastidious microorganism, from nasal-swab samples. The nPCR was able to identify up to three *M hyopneumoniae* strains within a single herd, two of them shared with other operationally related farms. We are aware that study of other genomic regions could have been useful to achieve a higher discrimination, but specific nPCRs have yet to be developed to allow *M hyopneumoniae* typing from nasal-swab samples, which are useful for monitoring live animals.

## Implications

- Typing *M hyopneumoniae* by an nPCR targeting the serine repeat motif of the *p146* gene is useful to identify several genotypes among pigs from one herd.
- *M hyopneumoniae* strains can remain in a herd in spite of the application of control measures.
- Unambiguous discrimination of *M hyopneumoniae* will require analysis of other genomic regions.
- Development and validation of nPCRs targeting other VNTR loci are needed to detect diversity of *M hyopneumoniae* from nasal-swab samples.
- Control measures for *M hyopneumoniae* eradication must be revised to identify reasons that could explain the failure of the eradication program used in these herds.


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### Conflict of interest

None reported.

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