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Short communication

Clonality of *Mycoplasma hyopneumoniae* in swine farms from Brazil

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ABSTRACT

Mycoplasma hyopneumoniae causes enzootic pneumonia (EP) in swine, a disease related to high economic losses in production systems. Epidemiological spread of *M. hyopneumoniae* clones was studied by multi-locus sequence typing (MLST) in several swine production regions but so far not in South America. Using MLST, we have therefore investigated *M. hyopneumoniae* clones circulating in farms from three main swine production regions in Brazil. Porcine lungs samples were collected between 2015 and 2016 in farms with EP outbreaks. Three geographically distant regions were selected, and 67 *M. hyopneumoniae* positive samples, each one from a different farm, were included in the study. The occurrence of five sequence types (ST) was demonstrated and the majority of the samples were identified as ST-69 (n = 60; 89.5%), followed by ST-70 (n = 3; 4.5%), ST-123 (n = 2; 3%), ST-124 (n = 1; 1.5%) and ST-127 (n = 1; 1.5%). There was no association of any specific ST with region or production system. The five STs were all new ones, probably representing unique Brazilian clones. ST-69 and ST-70 on one side and ST-123 and ST-124 on the other side are phylogenetically close, while ST-127 is singleton. In conclusion, our results showed a low variability and high clonality of *M. hyopneumoniae* genotypes from Brazilian farms affected by EP.

1. Introduction

Mycoplasma hyopneumoniae is the primary pathogen of porcine enzootic pneumonia (EP), a chronic respiratory disease associated with important economic losses in pig farms worldwide (Maes et al., 2008). This bacterium is usually transmitted by direct contact among pigs and by vertical transmission from dams to piglets (Calsamiglia and Pijoan, 2000; Fano et al., 2005). Pigs shed *M. hyopneumoniae* for long periods of time, thereby infecting susceptible animals (Pieters et al., 2009).

EP outbreaks have been investigated with different molecular typing techniques on *M. hyopneumoniae* isolates or directly in clinical material (Mayor et al., 2007, 2008). Conventional multi-locus sequence typing (MLST) has successfully been established for *M. hyopneumoniae* to evaluate epidemiological and phylogenetic relationships (Mayor et al., 2008; Kuhnert et al., 2011; Kuhnert and Overesch, 2014; Overesch and Kuhnert, 2017; Felde et al., 2018). Such method of genotyping has been very useful in field investigations because it has the great advantage of not requiring prior culture of this fastidious microorganism. While most MLST schemes use seven genes, it was also previously demonstrated that the use of only three (*rpoB*, *adk,* and *tpiA*) provides the same resolution as the complete scheme (Mayor et al., 2008; Felde et al., 2018). In addition, MLST is a robust, reproducible and intermediate scale typing approach for epidemiological and phylogenetic examinations of other *Mycoplasma* species infecting swine and other livestock animals, besides providing data easily comparable among laboratories through online databases (Tocqueville et al., 2014; Register et al., 2015; Dijkman et al., 2016; Ghanem and El-Gazzar, 2016; Bekő et al., 2019).

There are also other methods for *Mycoplasma hyopneumoniae* genotyping, such as pulsed field gel electrophoresis (PFGE), random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP) of the gene encoding lipoprotein P146, variable number of tandem repeats (VNTR) of the P97 encoding gene, and the multilocus VNTR analysis (MLVA) of the P146 and P97 genes (Stakenborg et al., 2005, 2006; Nathues et al., 2011). In Brazil, *M. hyopneumoniae* genotypes have already been analyzed with MLVA that

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demonstrated a high diversity within and among swine farms (Dos Santos et al., 2015; Pantoja et al., 2016; Takeuti et al., 2017). As other genotyping techniques were not used to analyze Brazilian samples yet, the present study aimed to genotype *M. hyopneumoniae* directly from lung samples using MLST, thereby improving epidemiological knowledge of the genetic characteristics of this bacterium in Brazil. Farms from the three main swine production regions in Brazil were investigated and the occurrence of five new sequence types (ST) was demonstrated with ST-69 being predominantly present in 89.5% of samples and found in all geographic regions.

2. Materials and methods

2.1. Samples

Porcine lungs samples were collected between 2015 and 2016. Veterinarians have collected lungs from animals with clinical signs of EP for further molecular diagnosis of *M. hyopneumoniae*. Bacterial DNA was extracted from the lungs tissues using the NewGene PreAmp and Prep Kit according to the supplier's instructions (Simbios Biotecnologia, Cachoeirinha, RS, Brazil). Real-time PCR was used for detection of *M. hyopneumoniae* with the NewGene MHYAmp kit according to the supplier's protocol (Simbios Biotecnologia). A total of 835 samples were thereby processed of which 321 were positive for *M. hyopneumoniae*. Among them, 67 DNA samples with a cycle threshold (CT) value < 30 in the real-time PCR were selected and subjected to MLST.

Pig farms with EP outbreaks were classified according to the production systems in: (a) breeding stocks (n = 1); (b) farrowing and nursery units (n = 4); and (c) fattening units (n = 60). There were also samples from two farms with closed herd system. Each sample represented one specific farm (totaling 67 farms), all of them located in three important pig producer-regions from Brazil: Midwest (states of Goiás and Mato Grosso), Southeast (state of Minas Gerais) and South (states of Paraná, Rio Grande do Sul and Santa Catarina) (Fig. 1).

2.2. Multi-locus sequence typing

Nucleotide sequence data of the genes *adk*, *rpoB*, and *tpiA* were obtained of the 67 *M. hyopneumoniae* positive samples by Sanger sequencing. Briefly, the three genes were amplified by primers and conditions described previously (Mayor et al., 2008). The amplified products were purified using NewGene Preamp kit according to the supplier's protocol (Simbios Biotecnologia), and sequenced on a Genetic Analyzer 3130 (Applied Biosystems, USA).

Sequences were edited and corrected in Sequencher (GeneCodes, Ann Arbor, MI, USA). Edited nucleotide sequences of the three genes of each sample were submitted and compared with the publicly available *M. hyopneumoniae* MLST database and new alleles and sequence types were deposited there (https://pubmlst.org/mhyopneumoniae/). All samples and sequences were further imported into Bionumerics v7.1 (Applied Maths, Sint-Martens-Latem, Belgium) and cluster analyses were carried out by UPGMA using individual similarity matrices from the three genes.

3. Results and discussion

In the present study, *M. hyopneumoniae* genotyping by MLST was performed for the first time on Brazilian samples of EP outbreaks from geographically unrelated farms. Brazil is one of the largest pig-producing countries worldwide. There are intensive swine producing farms located mainly in the Midwest, Southeast and South regions (Fig. 1). Therefore, using MLST, which has been regarded as a standard approach for genotyping bacteria, due to its good reproducibility and high discriminatory power (Felde et al., 2018; Bekő et al., 2019), it was possible to determine the alleles of the three analyzed genes and to detect the sequence type (ST) in a total of 67 lung samples. In the independent analysis of each gene, all *adk* alleles were already observed in previous studies, while for *rpoB* the new allele 58 was found and for *tpiA* the new allele 56 was identified. Based on new alleles and new allele combinations, the samples clustered into five new STs: ST-69 (n = 60; 89.5%), ST-70 (n = 3; 4.5%), ST-123 (n = 2; 3%), ST-124 (n = 1; 1.5%), and ST-127 (n = 1; 1.5%). Alleles and STs of the examined samples are summarized in Supplementary Table 1. Since all samples represented new STs based on the PubMLST database we hypothesize that they are specific Brazilian types.

In two samples, more than one ST seemed to be present since more than one allele was PCR amplified resulting in overlapping sequences with defined ambiguous positions (Supplementary Table 1). Like that, sample BRS032 had a single position in the *rpoB* sequence with multiple peaks, however this was compatible with presence of allele 33, and six ambiguous positions in the *tpiA* sequence, still compatible with allele 26. Similarly, sample BRS034 had one ambiguous position in the *adk* sequence, yet compatible with presence of allele 23 and 5 ambiguous positions in the *rpoB* sequence, but compatible with allele 33. These findings indicate that the most frequent ST-69 being also present in these two samples as well. For technical reasons a further four samples resulted in incomplete *rpoB* sequences missing 110 bp (BRS089, BRS093) and 15 bp (BRS085, BRS138). However, the available sequence indicated presence of allele 33 and thereby the most often found ST-69 also for these samples (Supplementary Table 1).

On the Brazilian farms tested ST-69 was predominant, without any relationship to geographic region, animal age or production system (Supplementary Table 1). Our results showed a low variability and high clonality of *M. hyopneumoniae* strains from Brazil by MLST. This low genetic variability was surprising when compared with previous MLST typing studies, which observed a high genetic variability on *M. hyopneumoniae* samples from different regions (Mayor et al., 2008; Kuhnert et al., 2011; Felde et al., 2018).

Although *M. hyopneumoniae* exhibits high strain variability, persistence of certain *M. hyopneumoniae* sequence types in operationally or geographically closely linked pig populations is not unexpected and it was already demonstrated in previous reports (Mayor et al., 2007, 2008; Overesch and Kuhnert, 2017). The samples of the present study were from geographically unrelated farms, with distances as big as 2300 km among them (Fig. 1). However, even with the great distance between sample origins, ST-69 was the predominant type in all sampling regions. In contrast, previous studies have reported a high diversity of *M. hyopneumoniae* Brazilian strains; nevertheless the results are not comparable since other genotyping approaches were used (Dos Santos et al., 2015; Pantoja et al., 2016; Takeuti et al., 2017).

ST-124 and ST-127 have a single (unique) detection, both from fattening farms. ST-124 was detected in South region, which interestingly has exclusively ST-69 in all other samples. ST-127 was identified in Southeast region. In this same region, there are another 11 farms, 9 (82%) with ST-69 and 2 (18%) with ST-70 (Supplementary Table 1). The South region is the biggest productive region of the country, and the farms are geographically closer than in other Brazilian regions. In addition, South farms are more linked, including animal genetics and food origins. Therefore, the low variability found among the 26 South samples could be associated to the operationally linked production sites.

Similarly, Midwest region has the biggest sample number (29/67; 43%), however, like as the other regions, ST-69 was the predominant type. Only two samples from Midwest were identified as others: one

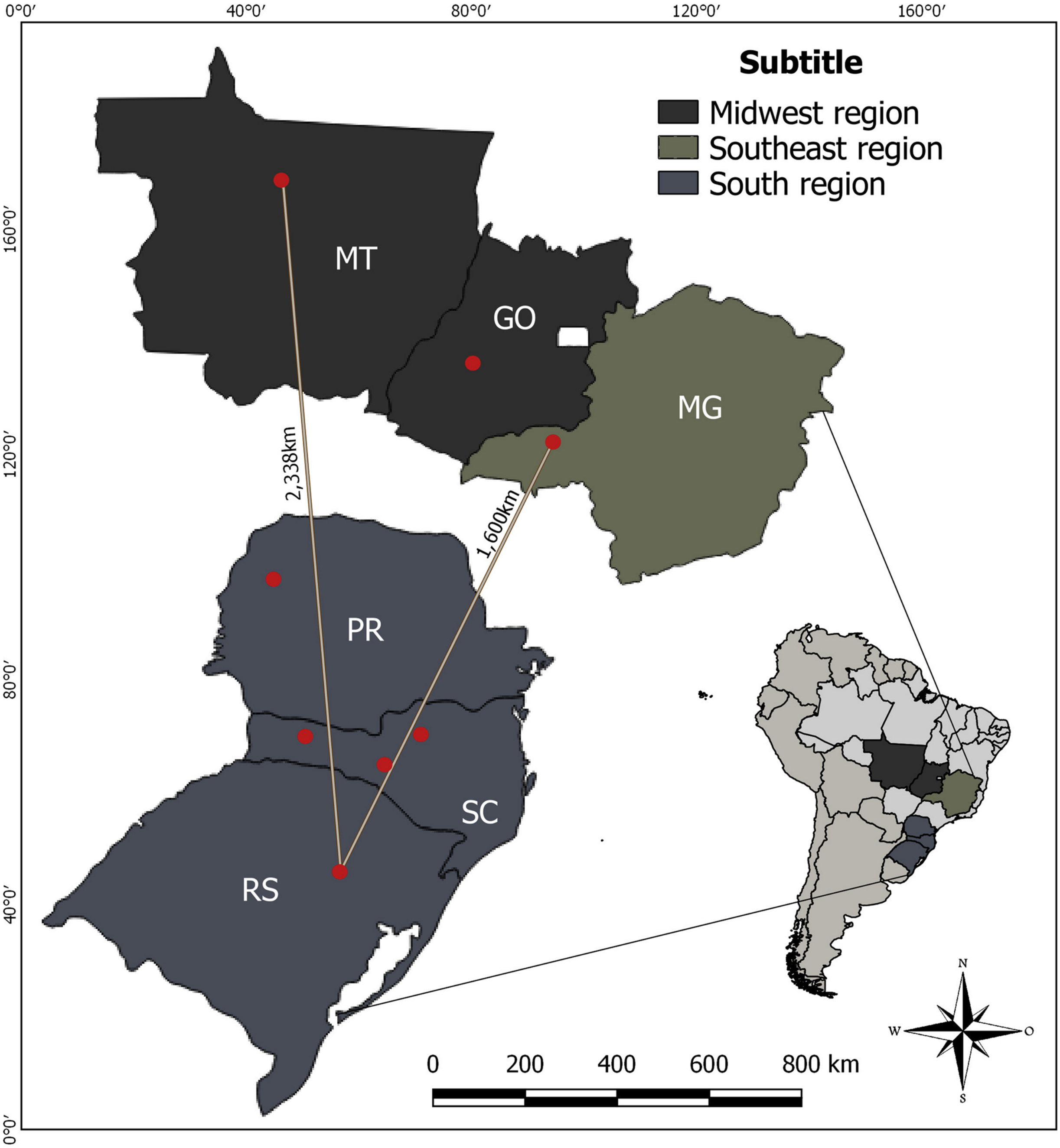

Fig. 1. Geographical distribution of pig farms sampled. Three Brazilian regions were studied. Red dots represents the farms city. Lines with Km highlight the distance between sampled farms. RS: Rio Grande do Sul, SC: Santa Catarina, PR: Paraná, MT: Mato Grosso, GO: Goiás, and MG: Minas Gerais.

sample was classified as ST-70, and one sample as ST-123.

ST-69 and ST-70 are phylogenetically close, differing only in one single base pair (bp) in the *adk* gene (Fig. 2). Interestingly, both are phylogenetically related to ST-41 representing the Brazilian strain 7422 but both differ already in *adk* (1 bp) and *rpoB* (2 bp) alleles from it. This reference strain was isolated 16 years ago from a South Brazil EP outbreak, and was classified as highly virulent strain (Siqueira et al., 2013). Similarly, ST-123 and ST-124 are closely related differing only in the *rpoB* allele with a 3 bp difference but forming a cluster of their own not much related to others (Fig. 2). The singleton represented by ST-127 differs at 6 bp in the *rpoB* allele from ST-70.

In conclusion, MLST revealed low diversity of *M. hyopneumoniae* from different regions but operationally linked production farms in Brazil. This first study on *M. hyopneumoniae* MLST genotypes in Brazilian will help to elucidate the epidemiology and possible sources and routes of *M. hyopneumoniae* strains to better control EP.

Declaration of Competing Interest

The authors declare that they have no competing interest.

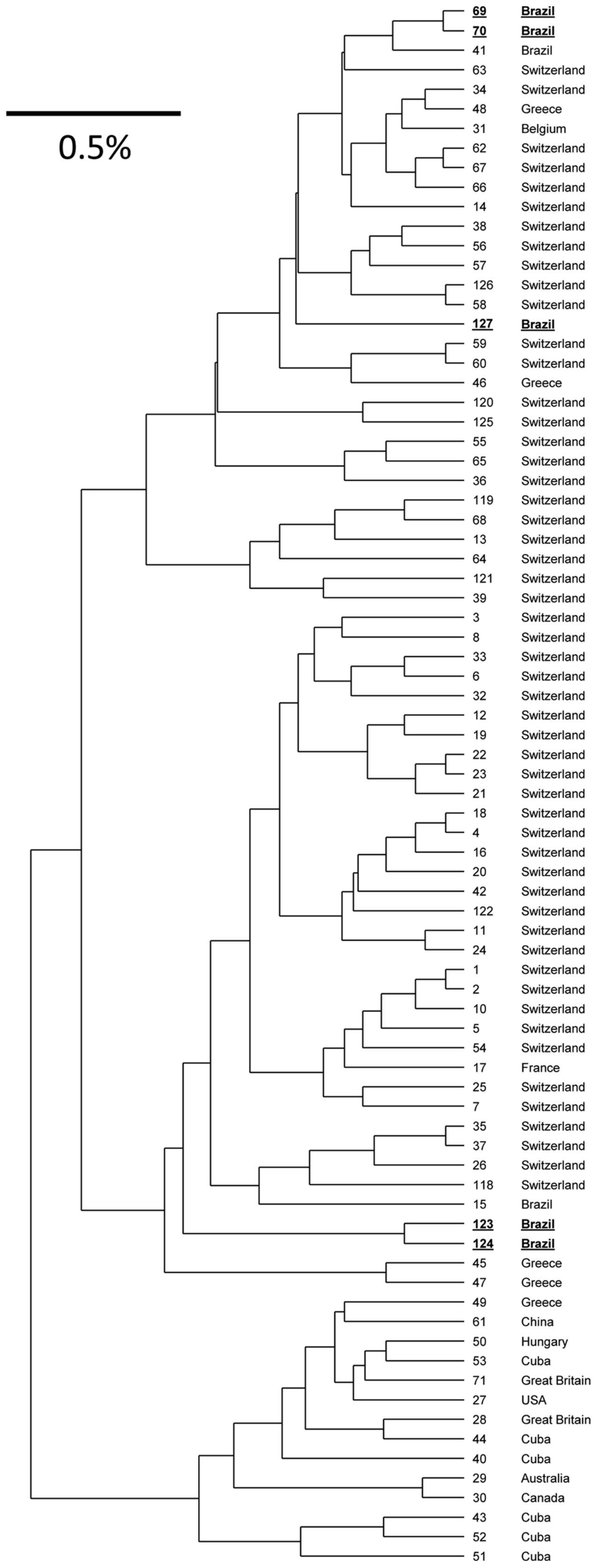

Fig. 2. Phylogenetic tree of selected sequence types (ST) based on *adk*, *rpoB* and *tpiA* sequences. STs determined in this study are indicated in bold underlined. Country origin of each ST are described. The bar represents the genetic difference within all three genes.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2019.108434.

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