LUCAS FERNANDO DOS SANTOS

INVESTIGATION OF GENETIC DIVERSITY IN MYCOPLASMA HYOPNEUMONIAE AND MYCOPLASMA HYORHINIS

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Medicina Veterinária, para obtenção do título de Doctor Scientiae

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APROVADA: 14 de agosto de 2015.

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RESUMO

SANTOS, Lucas Fernando dos, D.Sc., Universidade Federal de Viçosa, agosto de 2015. **Investigation of genetic diversity in Mycoplasma hyopneumoniae and Mycoplasma hyorhinis**. Orientador: Maria Aparecida Scatamburlo Moreira. Coorientadores: Maria G. Pieters e Srinand Sreevatsan

As duas espécies de Mycoplasma mais prevalentes na indústria suinícola são Mycoplasma hyopneumoniae e Mycoplasma hyorhinis. Estes patógenos podem causar perdas econômicas significativas para os produtores e para a indústria. Variabilidade genética tem sido observada em cepas de M. hyopneumoniae usando diferentes técnicas de tipificação. Já a diversidade genética de M. hyorhinis tem sido o foco de um número limitado de estudos nos últimos anos. Mycoplasma apresentam numerosas regiões repetitivas (VNTR) no seu genoma, e essas regiões repetitivas são conhecidos por serem sítios ativos para recombinação genética. Isso trouxe em evidencia a hipótese de que a análise em multilocus de repetições em Tandem de número variável (MLVA) seria uma técnica adequada para investigar a variação genética em Mycoplasma. Portanto, o objetivo principal desta dissertação foi investigar a distribuição não aleatória dos genótipos de diferentes localizações geográfica usando análise em multilocus de repetições em Tandem de número variável (MLVA) para tipificar M. hyopneumoniae e M. hyorhinis avançando o conhecimento sobre a diversidade genética e epidemiologia desses agentes. Quando o foco foi o estudo da diversidade genética de M. hyopneumoniae, um elevado número de tipos de MLVA parecem circular entre rebanhos de suínos com uma distribuição não aleatória dos tipos de MLVA entre regiões. Um único tipo comum não foi identificado entre as amostras obtidas a partir de todas as regiões em estudo. Do mesmo modo, foi observada a diversidade genética de M. hyorhinis, com uma variação limitada em um dos VNTRs alvo. Um ponto relevante observado neste estudo foi a identificação de diferentes tipos de MLVA no mesmo animal em diferentes tipos de amostras, o que sugere que o animal foi colonizado por diferentes cepas. Com foco na indústria de suínos do Brasil, uma das mais importantes do mundo, um estudo específico observou uma elevada diversidade de cepas de M. hyopneumoniae em circulação no país. A comparação entre o número de repetições em tandem (TR) no P97 RR1 e RR3 P146 mostrou uma correlação negativa significativa o que pode sugerir um possível mecanismo compensatório que permitiria a bactéria manter sua capacidade de aderência completa mesmo após a redução da TR em RR1-P97. Em conclusão, a identificação da

heterogeneidade genética de M. hyopneumoniae e M. hyorhinis ajudará investigações epidemiológicas ou de surtos locais e ajudará conceber estratégias de controle futuras, bem como servir como uma ferramenta potencial para o estudo da biologia evolutiva da espécie.

ABSTRACT

SANTOS, Lucas Fernando dos, D.Sc., Universidade Federal de Viçosa, August 2015. **Investigation of genetic diversity in Mycoplasma hyopneumoniae and Mycoplasma hyorhinis**. Adviser: Maria Aparecida Scatamburlo Moreira. Co-advisers: Maria G. Pieters and Srinand Sreevatsan

The two most prevalent Mycoplasmas species present in the swine industry are Mycoplasma hyopneumoniae and Mycoplasma hyorhinis. These pathogens can cause significant economic losses for producers and for the industry. Genetic variability has been observed in M. hyopneumoniae using different techniques and heterogeneity of M. hyorhinis has been the focus of a limited number of studies in the past years. The fact that Mycoplasma genomes contain numerous repetitive regions (VNTR) within their DNA and that they are known to be active sites for genetic recombination has prompted the hypothesis that Multiple locus variable number tandem repeat analysis (MLVA) would be an appropriate technique to investigate genetic variation in Mycoplasma. Therefore, the main goal of this dissertation was to investigate the non random distribution of genotypes from different geographical location using multiple locus variable tandem repeat analysis (MLVA) to type M. hyopneumoniae and M. hyorhinis and advance the knowledge on the genetic diversity and the epidemiology of these pathogens.When the M. hyopneumoniae study was taken in account, a high number of M. hyopneumoniae MLVA types appear to circulate among swine herds with a non-random distribution of the MLVA types among regions, also a common type was not identified among samples obtained from all regions in this study. Likewise, heterogeneity of M. hyorhinis was observed, with limited variation in one of the target VNTR in the M. hyorhinis study. A relevant point observed in this study was that different MLVA types were identified in the same animal in different sample types, which suggests that the pig was colonized with different strains. With a focus on Brazil pig industry,

one of the most important in the world, a specific study observed a high heterogeneity of M. hyopneumoniae strains circulating in the country, and the comparison between the number of tandem repeats (TR) in RR1 P97 and RR3 P146 showed a significant negative correlation that may suggest a possible compensatory mechanism that would allow the bacterium to keep its full adhesion capacity even after reduction of TR in RR1-P97. In conclusion, the identification of the genetic heterogeneity of M. hyopneumoniae and M. hyorhinis will assist local epidemiological or outbreak investigations, design future control strategies as well as serve as a potential tool to study the evolutionary biology of this species.

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General introduction

The two most prevalent Mycoplasmas species present in the swine industry are Mycoplasma hyopneumoniae and Mycoplasma hyorhinis. These pathogens can cause significant economic losses for producers and for the industry. The first one is related to the Enzootic Pneumonia (EP), a chronic respiratory disease. M. hyopneumoniae causes reduction in the growth rate, in the feed conversion rate, increase in medication costs, increased rates of mortality and depreciation of carcasses in the slaughterhouse due to the adherence of pleura and lung abscesses in interaction with other pathogens.

Despite the efforts and control strategies adopted, M. hyopneumoniae remains a widespread organism in swine populations, which makes the eradication of the disease a very difficult and frustrating task. Failure in the control strategies of EP could be associated to the strain variability considering that infection with one type of strain does not confer cross protection against an infection with different strain.

The second agent, M. hyorhinis, is considered an emerging pathogen, which are associated with polyserositis and arthritis in animals from 3 to 10 weeks of age. M. hyorhinis can be present in normal lungs, but it is more frequently found in pneumonic lungs, where it is considered a secondary agent.

Heterogeneity of M. hyorhinis strains in the swine populations could result in a difference in the dynamics of infection and spreading. The genetic diversity of M. hyorhinis has been the focus of a limited number of studies in the past years. Understanding the genetic diversity of M. hyorhinis could increase the understanding of the epidemiology in this bacterium.

Molecular typing methods have been described to investigate the genetic variability of M. hyopneumoniae and M. hyorhinis. However, these tools cannot be used effectively until they have been validated for each pathogen. A major purpose of the research described within this thesis was to investigate the non random distribution of genotypes from different geographical location using multiple locus variable tandem repeat analysis (MLVA) to type M. hyopneumoniae and M. hyorhinis. The MLVA method has been used to genotype several species of bacteria, including different Mycoplasma species. The use of MLVA has improved the understanding of bacterial epidemiology as it presents a high discriminatory power and can be an effective tool useful in outbreaks and epidemiological investigations. Other advantages for the MLVA include the ability to use genetic material without the need to grow the bacterium, to be performed directly from clinical specimens, and to be used as an initial high throughput screening and typing method for diagnostic laboratories. The fact that Mycoplasma genomes contain numerous repetitive regions within their DNA and that they are known to be active sites for genetic recombination has prompted the hypothesis that MLVA is an appropriate technique to investigate genetic variation in M. hyopneumoniae and M. hyorhinis.

Therefore, the main goal of this dissertation was to advance the knowledge on the genetic diversity and the epidemiology of M. hyopneumoniae and M. hyorhinis in swine populations. The results of this study will contribute to the better implementation of control and prevention strategies for both pathogens.

In Chapter I, a modified and standardized high-resolution multiple locus variable number tandem repeat analysis (MLVA) was used to investigate the genetic variability of M. hyopneumoniae circulating in the United States of America (USA), Brazil, Mexico and Spain. In Chapter II, the issue addressed was investigating the heterogeneity of M. hyorhinis using a

molecular tool based on the variable number of tandem repeats present in the genome of this bacterium. An MLVA assay was developed and applied to a strain collection for analysis of the diversity. And in the third chapter, the MLVA assay developed in the first chapter was applied to a set of samples from Brazil with the objective of describe the spatial distribution and genetic heterogeneity of M. hyopneumoniae in this country, and to investigate the association between number of tandem repeats in the two adhesion genes that were target in the MLVA assay (RR1-P97 and RR3-P146).

Literature Review

1.1 Mycoplasmas

Members of the Class Mollicutes, Mycoplasma belongs to a group of cell wall-less bacteria that infect a wide variety of plants and animals (including humans), Family Mycoplasmataceae and Genus Mycoplasma. They are small pleomorphic organisms whose diameter can vary from 0.2 to 0.3 µm (Razin et al., 1998), this allows the organism to pass through filters resulting in the contamination of cell culture (Kobish et al., 1996). Their morphology seems as "fried egg" colony shape, this can be explained by their total lack of cell wall (Razin et al., 1998). However some species have a cytoesqueleton, which is used to modulate and control their shape in cell division and the motility (Balish and Krause 2006; Razin et al., 2010). Mycoplasmas can replicate by binary fission like other prokaryotes (Razin et al., 2010). Usually, this organism is a surface parasite, although some species as Mycoplasma fermentans, Mycoplasma penetrans and Mycoplasma pneumoniae show intracellular location (Lo et al., 1993; Yavlovich et al., 2004 a; b) that may be considered a form of escape from the host immune system and from antibiotic action, resulting in the establishment of a latent or chronic infection state (Razin et al., 2010).

Mycoplasma presents a reduced genomic size that ranges from 580 kb to 1380 kb (Razin et al., 1998). The lack of genes involved in amino acid synthesis, making some species dependent of the host amino acid supplies (Himmelreich et al., 1996), which implies a limited biosynthetic capacity. For this reason, they become parasitic and obtain most of their nutrients from the host resulting in highly specific host and tissue range (Pollack et al., 1997; Hutchison

and Montague, 2002). In turn this can make mycoplasmas particularly difficult to culture as they have very specific growth requirements (Razin et al., 1998).

Mycoplasmas are facultative anaerobes that grow at 37^{o}C with 5-10 % of CO_2 and need special media with growth requirements when cultured in vitro. They can be found on mucosal surfaces of the conjunctive, nasal cavity, genital and intestinal tracts of animals and humans. Mycoplasmas can be differentiated by colonial morphology, requirements for cholesterol, biochemical reactivity and host specificity. The hosts and disease conditions are shown in Table 1.

Table 1 - Major diseases associated with mycoplasma species of veterinary significance, the host and the disease conditions which they cause.

Mycoplasma Species	Hosts	Disease conditions
M. mycoides subsp. mycoides	Cattle	Contagious bovine pleuropneumonia
M. bovis	Cattle	Mastitis, pneumonia, arthritis
M. agalactiae	Sheep, Goats	Contagious agalactia
M. capricolum subsp. capripneumoniae	Goats	Contagious caprine pleuropneumoniae
M. capricolum subsp. capricolum	Sheep, Goats	Septicaemia, mastitis , polyarthritis, pneumonia
M. mycoides subsp. capri	Goats, Sheep	Septicaemia, pleuropneumonia, arthritis, mastitis
M. hyopneumoniae	Pigs	Enzootic pneumonia
M. hyorhinis	Pigs (3-10 weeks of age)	Polyserositis and arthiritis
M. hyosynoviae	Pigs (10-30 weeks of age)	Polyarthritis

M. gallisepticum	Chickens Turkeys	Chronic respiratory disease Infectious sinusitis
M. synoviae	Chickens , Turkeys	Infectious synovitis
M. meleagridis	Turkeys	Airsacculitis, bone deformities, reduced hatchability and growth rate
M. haemofelis	Cats	Feline infectious anemia

Adapted by Quinn et al., 2011

1.2 Importance of Mycoplasma in porcine respiratory diseases complex

During the last decades pork production has enhanced substantially. As consequence of the increase in herd size and the improvements of infectious disease diagnostic, the identification of infectious diseases has become consistently. Respiratory disease in swine is the most important health concern for swine producers and has been identified as the greatest causes of economic losses in pig production (Opriessnig et al., 2011). These losses are represented by increased spending on medicines, losses in animal performance and carcasses at slaughterhouses (Sorense et al., 2006; Martínez et al., 2007).

With the improvement in the pathogen detection methods was possible to identify a multiple microbial infections causing respiratory disease in pigs. This polymicrobial infection is often the result of a combination of primary and opportunistic infectious agents (Opriessnig et al., 2011). To the multiple etiologies pneumoniae was created the term porcine respiratory disease complex - PRDC (Halbur, 1997). Slow growth, decreased feed efficiency, lethargy, anorexia, fever, cough, and dyspnea are symptoms of PRDC (Holko et al., 2004). Pneumonia and pleuritis are the most frequent lung lesions observed at the slaughterhouse as consequence of PRDC, with a

prevalence of pneumonia ranging from 19% to 79% and for pleuritis from 3.8% to 62% (Wilson et al., 1986; Hartley et al., 1988; Enoe et al., 2002; Leneveu et al., 2005; Fraile et al., 2010; Meyns et al., 2011). The severity of PRDC manifestation in pigs are direct related to the interactions and synergy of infectious factors (viral and bacterial pathogens), environmental factors, type of production system and management, and genetics, age and immunological status of the animals. Different bacteria and viruses are involved in the development of PRDC. Mycoplasma hyopneumoniae (M. hyopneumoniae), Pasteurella multocida (P. multocida), Actinobacillus pleuropneumoniae (A. pleuropneumoniae) which can act as primary bacterial respiratory pathogens; viral respiratory pathogens as swine influenza viruses (SIV), porcine reproductive and respiratory syndrome virus (PRRSV) and porcine circovirus type 2 (PCV2) and opportunistic bacterial respiratory pathogens as Streptococcus suis (S. suis) and Haemophilus parasuis (Hps) (Holko et al., 2004; Sorensen et al., 2006; Opriessnig et al., 2011; Savic et al.,2015).

The importance of the Mycoplasmas in the respiratory diseases is focused in the M. hyopneumoniae. M. hyopneumoniae is especially important for PRDC because infects the cilia of the epithelial cells of the respiratory tract resulting in destruction of the cilia (DeBey and Ross, 1994; Young et al., 2000). Mucociliary apparatus are an important mechanism used as host defense mechanism to move foreign materials out of the airways. The damage and loss of the cilia results the increased incidence of secondary bacterial infections associated with M. hyopneumoniae infection (Thacker and Thanawongnuwech, 2002; Opriessnig et al., 2011). Studies indicate that other Mycoplasma can be important in PRDC, M. hyorhinis. M. hyorhinis appears to be frequently associated with M. hyopneumoniae in PRDC cases (Thacker, 2006; Lin et al., 2006; Hansen et al., 2010), but more research in this area is needed.

1.3 Porcine Mycoplasmas highlighting Mycoplasma hyopneumoniae and Mycoplasma hyorhinis

Infections by Mycoplasmas represent one of major economic losses in animals. Mycoplasma can infect the respiratory tract especially in pigs, ruminants and poultry (Table 1). Some mycoplasmas have been isolated from pigs and they have been implicated in respiratory and systemic diseases. Mycoplasma hyopneumoniae is the primary agent of enzootic pneumonia (EP) in pigs, a chronic disease that affects the growth performance, characterized by high morbidity and low mortality and increased susceptibility to co-infections for other pathogens (Ciprian et al., 1988). Also M. hyopneumoniae is considered one of the primary agents involved in respiratory disease complex in pigs (Thacker et al., 2006). This infection is highly prevalent where causing significant economic losses in pig production systems (Ross et al., 1999). M. hyorhinis is a common inhabitant of the respiratory tract of pigs and can cause polyserositis in animals from 3- 10 weeks of age when it becomes systemic. The mechanism that M. hyorhinis uses to become systemic is unclear, however the first step of the colonization is attachment to the cilia in the upper respiratory tract (Gois et al., 1974). Also, M. hyorhinis can be present in cell culture lines as contaminant (Kobish et al., 1996).

Another mycoplasma, M. hyosynoviae, affects pigs from 10 to 30 weeks of age and has an affinity for synovial tissue (Kobish et al., 1996). The sow is the main source of infection of the piglets, which appear resistant in young ages (Ross et al., 1971). M. hyosynoviae spreads hematogenously and presents a special affinity to joint synovial tissues (Ross et al., 1971). High morbidity rates range between 10 – 50% among grower and finisher animals and gilts (Kobish et al., 1996). Polyarthritis is a common problem in young breeding animals and grower finisher animals and the treatment cost of the enzootic arthritis can increase the production costs (Nielsen

et al., 2001). In general, the clinical signs observed are difficulty in moving, stiffness, lameness, arched backs and inability to get up (Kobish and Friis , 1996). Mycoplasma suis, a hemotropic mycoplasma that parasitizes the surface of erythrocytes and causes porcine eperythrozoonosis, an immune mediated hemolytic anemia (Messick, 2004). This can affect young and growing pigs, causing disease characterized by listlessness, fever, anorexia, hemolytic anemia and icterus (Hoelzle, 2008). Infected animals can be subclinical or a chronically infected carrier and spread the disease in the herd or apparently normal for months before developing the symptoms (Hoelzle, 2008). M. suis suppresses the host immune system, increasing the susceptibility to other pathogens (Zachary and Smith, 1985). Mycoplasma flocculare is present in the nasal cavities, and is similar to M. hyopneumoniae but there is no diseases related with this pathogen (Kobish and Friis, 1996). M. flocculare can attached to the cilia but is different from M. hyopneumoniae, as it does not cause destructions of the epithelium (Young et al.,2000). Siqueira et al., (2013) found that despite M. flocculare and M. hyopneumoniae displaying a high degree of similarity, the structure and organization of genes that encode adhesins exhibit differences in the sequence for important domains for adhesion to host cells.

Mycoplasma hyopneumoniae

M. hyopneumoniae belongs to the class Mollicutes. Like all member of this class, M. hyopneumoniae has no cell wall, presents a reduced genome and a low G+C content (Razin et al., 1998). So far, the whole genome of six strains has been sequenced (Minion et al., 2004; Vasconcelos et al., 2005; Liu et al., 2011; Siqueira et al., 2013). The genome size ranges from 892 to 926 kb with a G+C content of 28 %. Like most Mycoplasma, M. hyopneumoniae codon usage differs from the universal genetic code. The UGA codon encodes for tryptophan instead of recognizing it as a stop codon.

The bacterium was isolated for the first time in 1965 by Mare et al (1965) and was identified as M. hyopneumoniae. Other research group led by Goodwin isolated the microorganism and named it Mycoplasma suipneumoniae (Goodwin et al., 1967) EP was reproduced experimentally showing the pathogenicity of the microorganism. Later, it was shown that both isolates were the same and the bacterium was named M. hyopneumoniae.

The M. hyopneumoniae is spread worldwide especially in the countries where the swine industry is important (Sibila et al., 2004; Maes et al., 2008). Studies in different countries indicate that lesions suggestive of EP occur in 30 to 80 % of slaughtered pigs. In southern Brazil, which accounts for about 80 % of the national slaughter, it was found that 55 % of pigs had lesion suggestive of EP and 100 % of herds examined were affected by M. hyopneumoniae (Sobestiansky, 1999). Maes et al, (1999) reported that over 90 % of Belgian herd were infected with M. hyopneumoniae. Introduction of M. hyopneumoniae in a naive herd is caused mainly by airborne transmission or by introduction of subclinical infected animals (Sibila et al., 2009). Several risk factors are associated to (re) infection of a swine herd with M. hyopneumoniae. In endemic regions, replacement of boars and gilts as part of the restocking policy, the distance to neighboring pig farms and the distance to the next countryside highway can increase the risk of a herd become infected (Maes et al., 2000).

The persistence and maintenance of the M. hyopneumoniae in infected herds may be related to three factors. 1 - When transmission occurs from infected sows to their offsprings, which in general may occurs in the first week of age (Sibila et al., 2007; Holst et al., 2013). Pieters et al. (2014) demonstrate that the prevalence of M. hyopneumoniae at weaning increased with piglet's age if at least one dam was M. hyopneumoniae positive. 2 - by transmission between infected animals to littermates and pen-mates at the farrowing or nursery sites. 3 - by

transmission between older pigs (growing or finisher animals) to younger piglets that enter in the facilities without all in all out protocol (Ross, 1999; Casamiglia and Pijoan 2000, Rautianen and Wallgreen, 2001). Gilts and sows with low parity have more chance to transmit the pathogen to their offspring (Fano et al., 2007), but sow with higher parity, up to the 7th parity, also may shedding the organisms (Calsamiglia and Pijoan, 2000). M. hyopneumoniae infected pigs can remain infectious and be a reservatory of the organism for at least 214 days post infection (Pieters et al., 2009). The transmission rate of M. hyopneumoniae between pigs is slower compared to viral infections such as swine influenza. One M. hyopneumoniae positive pig at weaning will transmit to one pen mate during a nursery period of 6 weeks (Meyns et al., 2004; Villarreal et al., 2011). The dynamics of infections and clinical implication may vary according to the type of production system (Sibila et al, 2004).

Infection occurs via the respiratory route and the incubation period may vary from one day to 10 months, with an average of five weeks (Sobestiansky, 1999). The pathogenesis of the disease caused by M. hyopneumoniae depends on adherence of these microorganisms to the host lung ciliated epithelium (Wannemuehler & Galvin, 1994).

Although virulence factors of M. hyopneumoniae have not been well characterized, the adherence to the respiratory epithelium is recognized as an important step for colonization and infection (Ross, 1999). M. hyopneumoniae infects epithelial cell of the trachea, bronchi and bronchioles, attaching to the cilia and promoting a reduction in ciliary activity, with damage and loss of cilia (Debey and Ross, 1994). Thus, the loss in efficiency of the mucociliary system, an important non-specific defense mechanism and an increase in mucus production favors secondary infections (Ciprian et al., 1988; Debey and Ross, 1994; Sorensen et al., 1997). The physical location of this organism in the surface walls of the cilia and epithelial helps escape the

host immune system and interfere to the effectiveness of antimicrobial treatment for hindering their release into the site of infection (Schwartz, 2001). M. hyopneumoniae interacts with alveolar macrophages and lymphocytes, stimulating them to produce proinflammatory cytokines (TNF-α, IL-1 and IL-6) being responsible for lung injury by perivascular and peribronchial lymphoid hyperplasia (Rodriguez et al., 2004). The advancement results in the peribronchial lymphoid hyperplasia are the obstruction of airway, leading to the formation of atelectatic lung lesions, with coloration varies from dark red to purple in acute cases to grayish brown in chronic cases (Thacker and Minion, 2012), located mainly in the cranioventral region of apical and cardiac lobes of the lung (Sobestiansky et al., 1999).

Although the isolation of M. hyopneumoniae is the "gold standard" diagnostic, this microorganism is very difficult to be isolated and identified as they require special growth medium and may take 4 to 8 weeks to multiply. Furthermore, successful isolation of M. hyopneumoniae may be difficult due to the presence of other mycoplasmas with rapid growth, such as M. hyorhinis, making diagnosis via cultivation sometimes not feasible (Thacker, 2006). As a result, bacterial isolation is not used as a routine diagnostic (Sibila et al., 2009).

Serological tests are the most commonly used tool to diagnose EP. Detection of antibodies against M. hyopneumoniae by ELISA may be carried out, and less frequently by the complement fixation test. ELISA is a quick and inexpensive automated method that provides useful information about the presence of maternally derived or acquired antibodies (Sibila et al., 2009). Currently, the tests used are blocking ELISA and indirect ELISA. However, comparative studies have reported differences in terms of sensitivity and specificity of these kits (Sorensen et al., 1997; Thacker, 2004, Gomes Neto et al, 2014).

The fluorescent antibody technique (FA) and immunohistochemistry (IHC) are used for identification of M. hyopneumoniae in tissues or swab blades. However, these techniques have the disadvantage of only be done post mortem (Sibila et al., 2009).

The accurate detection of M. hyopneumoniae increased significantly with advances in molecular biology methods based on the application of recombinant DNA technology, such as polymerase chain reaction (PCR). Since then, several tests have been developed (Stark et al, 1998; Calsamiglia et al., 1999; Caron et al., 2000; Verdin et al., 2000; Kurth et al, 2002; Thacker, 2004; Strait et al., 2008).

The strain variability of M. hyopneumoniae has been suspected for a long time and has been the main focus of several studies (Ro and Ross, 1983; Zielinski and Ross, 1990; Frey et al., 1992; Artiushin and Minion, 1996). Frey et al. (1992) observed the existence of different strains with differences in virulence by chromosomal restriction patterns. High variation in virulence was observed between M. hyopneumoniae strains isolated from different swine herds. A fragment of 5kb in RAPD analysis was associated with highly and moderately virulent strains, and interestingly this fragment was not observed in low virulent strains (Vicca et al., 2003). Differences in pathogenicity between the highly and low virulent isolates was associated with a faster in vitro growth, a higher capacity to multiply in the lungs and the induction of a more severe inflammation process by the highly virulent isolate (Meyns et al., 2007).

Recent studies using different typing methods to analyze genetic diversity of M. hyopneumoniae, including amplified fragment length polymorphism (AFLP), pulse-field gel electrophoresis (PFGE), multi-locus sequence typing (MLST), random amplified polymorphic DNA (RAPD) analysis, restriction fragment length polymorphism (RFLP) and Mulitple locus of variable number of tandem repeat analysis (MLVA) have been applied to variable sets of M.

hyopneumoniae strains from different sources (Stakenborg et al., 2005; Stakenborg et al., 2006; Mayor et al., 2007; Mayor et al., 2008; Vranckx, et al., 2011; Charlebois et al., 2014). It is clear that even with several studies using different typing methods being performed on the investigation of genetic diversity of M. hyopneumoniae, many aspects remain unknown. The high diversity between strains from different herds and the difficulties in showing the association between the genotype and the virulence of the strain still unclear.

Mycoplasma hyorhinis

M. hyorhinis is a swine pathogen (Ross et al., 1973) and it is usually found as a contaminant of mammalian tissue cultures. M. hyorhinis was first isolate by Switzer (1953), and was considered to be the cause of enzootic pneumonia, which was later correctly then associated to M. hyopneumoniae (Mare and Switzer, 1965; Goodwin et al., 1967). M. hyorhinis play a role as an obligate parasite of swine, and can cause disease as a secondary pathogen. Possible features that distinguish field isolates from tissue culture strains have only recently begun to be explored at the genome level. To date, five strains had genomes completed sequence (Liu et al., 2010; Kornspan et al., 2011; Calcutt et al., 2012; Goodison et al., 2013; Dabrazhynetskaya et al., 2014). The completed genome range from 830 to 840 kb with an average G+C content of about 26 %. Even with their small genome, these organisms are able to survive on the host, producing the metabolics needed for their growth and escape from the host defense system. Chromosomal gene rearrangement is one strategy used by mycoplasmas to achieve these tasks (Bork et al., 1995).

M. hyorhinis is commonly present in the respiratory tract of pigs (Switzer, 1955). M. hyorhinis can be present in normal lungs, but it is more frequently found in pneumonic lungs, where it is considered a secondary pathogen (Kobayashi et al., 1996; Kawashima et al., 1996;

Lin et al., 2006). M. hyorhinis can cause polyserositis and arthritis in animals from 3 to 10 weeks of age when it becomes systemic (Potgieter and Ross, 1972; Friis and Feestra, 1994). This bacterium has affinity for serosal surfaces and may lead to acute inflammation of the serosa of body cavities and joint.

An increase in the detection of M. hyorhinis as an etiological agent of polyserositis and arthritis in piglets in recent years demonstrates the relevance of this pathogen in the swine industry as an emerging challenge (Rovira et al., 2010).

Most of swine producing countries worldwide have reported the presence of M. hyorhinis associated with clinical disease. However, most studies that investigate the epidemiology of this pathogen were done in slaughter pigs or in pigs submitted to diagnostic laboratories, which could bias the analyses of the epidemiology of M. hyorhinis in swine farms (Rovira et al 2010).

A small proportion of piglets become colonized with this microorganism by contact with the dams and later nose-to-nose contact among pigs in the nursery increases transmission among pen mates (Kobish and Friis, 1996; Clavijo et al., 2012). In a longitudinal study, Clavijo et al (2012) evaluated by PCR of nasal swab the dynamics of colonization by M. hyorhinis in farms with polyserositis history. Neonatal piglets had 1.7 % of M. hyorhinis positivity and an increasing in the percentage of positivity of 85 % was observed after weaning. Palzer et al (2008) found that 80 % of pneumonic lungs and 37 % of normal lungs from slaughter pigs were positive for M. hyorhinis by PCR in Germany.

In addition to infect pigs, M. hyorhinis has the ability to infect tumor cells or cell culture. The infection of M. hyorhinis in benign tumors can cause phenotypic changes and differential gene expression leading to malignant transformation of these tumors, as demonstrated by the

ability of this agent to infect gastric tumor cells and prostate (Urbanek et al, 2011;. Xu et al, 2013).

To date no virulence factors of M. hyorhinis have been described. However, diverse studies have been showed the differences in virulence between isolates (Gois and Kuksa, 1974; Lin et al., 2006; Ross and Switzer, 1963).

Experimental studies have shown that immune suppressive factors make the colonization by M. hyorhinis more intense in the respiratory tract and serosa. It is noted that in elderly and immuno competent animals frequently occur the development of arthritis (Magnusson et al., 1998). Observations under field conditions showed increased diagnosis of M. hyorhinis in cases of coinfection (PCV2 and PRRSV), suggesting that viral agents are immunosuppressive and facilitate colonization and spread of M. hyorhinis in the host (Kawashima et al., 1996; Kobayashi et al., 1996; Kawashima et al., 2007; Kixmöller et al., 2008).

In most infections, animals show mainly fever, dyspnea, and reluctance to move (Kobisch and Friis, 1996). Infection in pigs is usually characterized by arthritis and polyserositis but most of the infections are subclinical (Potgieter and Ross, 1972). Other clinical presentations associated with M. hyorhinis include rhinitis, pneumonia, otitis, conjunctivitis and abortions (Friis, 1971; Poland et al., 1971; Morita et al., 1993; Morita et al., 1999; Shin et al., 2003; Thacker and Minion, 2012).

The isolation of M. hyorhinis is less laborious as compared to M. hyopneumoniae, however, it is not frequently used in routine. The analysis of clinical signs and gross lesions represents a good strategy to diagnostic M. hyorhinis. However, different etiologies can causes polyserositis, like Haemophillus parasuis and Streptococcus suis. This facts is should take into account for a confirmatory diagnostic of polyserositis caused by M. hyorhinis (Kang et al.,

2012). Techniques like immunohistochemistry and immunofluorescence had been used to detect the presence of M. hyorhinis in the tissues (L'Ecuyer and Boulanger, 1970; Gois et al., 1971; Potgieter and Ross, 1972). Several PCR based diagnostic tests have been used to identify M .hyorhinis (Stemke et al., 1994; Kobayashi et al., 1996; Caron et al., 2000; Lin et al., 2006; Timenetsky et al., 2006; Kang et al., 2012; Tocqueville et al., 2014, Clavijo et al., 2014) however, little is known about the sensitivity and specificity. Real-time PCR is the most sensitive assay available to detect and quantify M. hyorhinis and can be conducted directly on clinical specimens (such as joint fluid), joint swabs, nasal swabs, or tonsil scrapings. Currently, there are no immunological tests commercially available for M. hyorhinis.

An important feature of mycoplasmas is the ability to change their surface antigens. Through this mechanism, these microorganisms are able to evade the immune response mounted by the host and establish a chronic infection (Muneta et al, 2008). When compared to other mycoplasmas, M. hyorhinis exhibits an aggressive growth due to differences found in components involved in metabolism and suggestive of evasion of the host immune system (Siqueira et al., 2013). The occurrence of variation in the expression of surface lipoproteins in M. hyorhinis resulted in the generation of population diversity (Rosengarten and Wise, 1991). It has been suggested that the source of genetic variation in the variable lipoprotein (Vlp) is the insertion and deletion of tandem repeats (VNTR) within the 3'region in the Vlp genes (Rosengarten and Wise, 1991; Yogev et al., 1991; Citti et al., 2000).

The heterogeneity of M. hyorhinis has been the focus of a limited number of studies in the past years. Restriction endonuclease analysis was applied by Darai and collegues (1982) in a strain collection of M. hyorhinis to verify the characteristic of fragment pattern of DNA bands. More recently, two typing methods were used to identify the genetic diversity in M. hyorhinis,

namely sequencing of the p37 gene and a multilocus sequencing typing (MLST; Tocqueville et al., 2014).

1.4 Mycoplasmas & Variable number of tandem repeat (VNTR)

The surface of the Mycoplasma membrane establish a critical role in the infection process, due the importance of this component in mediating the basic functions, such as host cell interactions, transport of nutrients and host immune defenses (Citti et al.,2010). A sets of related genes are responsible to encoding surface lipoproteins and these genes are susceptible to high-frequency, reversible mutations that could lead variable expression patterns of corresponding gene products (Razin et al., 1998). Variation of lipoproteins would results in a modification of structures at the bacterial cell surface which could be involved in antigenicity (Rosengarten et al., 2000). Lipoprotein variations has been described in many Mycoplasmas species and are responsible to generate surface antigen diversity which could be result of phase and/or size variation (Rocha and Blanchard, 2002). Genomic repeats, such as tandem repeats constitute source of these variation which occurs in recombination events.

Mycoplasmas contain various repeated sequences, which could be structured in tandem, in their genome. The variation of the number of the repeat sequence in one gene could lead a size variation of expressed lipoproteins genes (Citti et al.,2010). Functions of these VNTRs are partially understood, however, appear that can shield the mycoplasma cells from complement and modulate biofilm formation (Simmons et al., 2007).

Size variation of surface proteins has been reported in different Mycoplasmas species, such as, M. hyorhinis (Vlp); M. pulmonis (Vsa) and M. bovis (Vsp) (Behrens et al., 1994; Citti et

al.,2000; Rocha and Blanchard, 2002). M. hyorhinis the size variation of the Vlp gene occurs in high frequency (Rosengarten and Wise, 1991). The expression of the Vlp genes is controlled by variation in the size of the polyadenosine tract, the length of 17 polyadenosine tract is necessary for the gene transcription (Yogev et al.,1991; Citti and Wise, 1995). Longer variants of the Vlp genes increase resistance to growth inhibitory antibody (Citti et al., 1997). In M. pulmonis, the expression of Vsa proteins with high number of VNTRs concedes protection against killing by complement and instigates formation of microcolonies. However, stimulation of the cytoadherence are achieved with the expression of the VSA proteins with few VNTRs (Simmons and Dybvig, 2003; Simmons et al., 2004). Also, the lenght of the Vsa proteins is correlated with the thickness of the surface of the M. pulmonis. More repeats the Vsa protein presents, more thick it will be (Simmons and Dybvig, 2003).

Expressions of lipoproteins encoded by single copy gene are subject to high frequency phase variation and the control of the expression is achieved by variation in the length of repeated sequence. An example is in M. hominis, which the variation in the VNTRs results in difference in the adherence (Zhang and Wise, 1997).

1.5 Molecular technique to achieve the VNTRs

The molecular detection of a pathogen plays a key role in the identification, typing and association or such pathogens with disease. Different molecular typing methods can be used to investigate outbreaks and the epidemiology of bacterial pathogens (Sabat et al., 2013). Most of mycoplasmal diseases need the specific laboratory diagnostic strategies to identify and type the pathogen and the strategies are quite different than those for fast-growing bacteria due the specific requirements of mycoplasmas to growing in vitro.

Several different typing techniques of mycoplasma have been developed, each one of which has its advantages and limitations with respect to cost, time, reliability, specificity, and sensitivity. A selection of typing methods that were used to date and may be usefully applied to M. hyopneumoniae and M. hyorhinis are shown in table 2.

The fact that mycoplasma genomes contain numerous repetitive regions within their DNA and that they are known to be active sites for genetic recombination has prompted the hypothesis that MLVA is an appropriate technique to investigate genetic variation in M. hyopneumoniae and M. hyorhinis. MLVA analysis is a feasible and suitable method to characterize bacteria without the necessity of any culture steps. It was reported as being a useful tool for typing different mycoplasmas. It was demonstrated that specific VNTRs are numerous in the genome of mycoplasmas and could be associated to functional properties (Hsu and Minion, 1998). These facts might approve MLVA as the method of choice when it is necessary to characterize a large numbers of mycoplasmas strains. Therefore, the objective of this thesis was to investigate the nonrandom distribution of genotypes of M. hyopneumoniae and M. hyorhinis using the MLVA approaches.

Table 2 – Summary of molecular techniques used to genotype M. hyopneumoniae and M. hyorhinis.

Organism	Typing method	Target/gene	Reference
Mycoplasma hyopneumoniae	PFGE	ApaI, Sal I, ApaL, Asp718	Stakenborg et al., 2005
	AFLP		Kokotovic et al., 1999

			Stakenborg et al., 2006a
	RAPD - PCR	OPA-3 primer	Artiushin and Minion 1996 Vicca et al., 2003 Stakenborg et al., 2006a Nathues et al., 2011
	PCR - RFLP	P146	Stakenborg et al., 2006a
	PCR - Sequencing	P97 P146	Wilton et al., 1998 Mayor et al., 2007a
	MLST	adk, rpoB, tpiA	Mayor et al., 2007b
	VNTR	P97 VNTR genes P97, P146, H4	Stakenborg et al., 2006a De Castro et al., 2006 Nathues et al., 2011
	MLVA	Variable number of tandem repeats genes	Vranckx et al., 2011; Charlebois et al., 2014.
Mycoplasma hyorhinis	REA		Darai et al., 1981
	PCR-Sequencing	P37	Tocqueville et al., 2014
	MLST	dnaA, rpoB, gyrB, gltX, adk, and gmk.	Tocqueville et al., 2014

PCR, polymerase chain reaction; RAPD, randomly amplified polymorphic DNA; VNTR, variable number tandem repeats; AFLP, amplified fragment length polymorphism; RLFP, restriction fragment length polymorphism; PFGE, pulsed-field gel electrophoresis; REA, restriction endonuclease analysis; MLST, multi locus sequence typing; MLVA, multiple locus of variable number of tandem repeat analysis.

1.5 Aims of this study

A high strain diversity of M. hyopneumoniae and M .hyorhinis has been observed. Differences in strains within and among swine populations could impact the observed dynamics of infection and spread. The genetic diversity within a herd, the clinical importance of this diversity as well as the persistence of different strains in pigs under field conditions are not known.

Our hypothesis is that a nonrandom distribution of genotypes of M. hyopneumoniae and M. hyorhinis strain vary according to distinct geographical regions and that those strains differences account for the different clinical presentation and diverse response to control measures observed in the field. The specific objectives are:

- Standardize a highly sensitive and discriminatory technique (Multiple-Locus Variable-Number Tandem-Repeat Analysis (MLVA)) for differentiation of M. hyopneumoniae strains.
- Investigate the genetic variability of M. hyopneumoniae strains in clinically relevant specimens from geographically distinct regions

- Standardize a highly sensitive, highly discriminatory technique (Multiple-Locus Variable-Number Tandem-Repeat Analysis (MLVA)) for differentiation of M. hyorhinis strains.
- Investigate the genetic variability of M. hyorhinis strains in clinically relevant specimens from geographically distinct US farms regions

Such information will contribute to the better understanding of the epidemiology of both pathogens, which can help the implementation of control and prevention strategies.

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CHAPTER I - Genotype distribution of Mycoplasma hyopneumoniae in swine herds from different geographical regions

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Title Page

Short Communication

Genotype distribution of Mycoplasma hyopneumoniae in swine herds from different geographical regions

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Abstract

Genetic heterogeneity of Mycoplasma hyopneumoniae in pigs has been reported, however there has been limited reproducibility on the molecular methods employed so far. The aim of this study was to modify and standardize a high-resolution multiple locus variable number tandem repeat analysis (MLVA), to investigate the genetic variability of M. hyopneumoniae circulating in the United States of America (USA), Brazil, Mexico and Spain. The MLVA was standardized on the basis of the number of tandem repeats in two Mycoplasma adhesins, P97 and P146, which are proteins involved in the adherence of the pathogen to cilia. A total of 355 samples obtained from the four countries were analyzed. The Simpson's diversity index for the assay was D = 0.976 when samples from all countries were combined. A large number of MLVA types (n = 139) were identified, suggesting that multiple M. hyopneumoniae variants are circulating in swine. The locus P97 had 17 different types with 2–18 repeats. The P146 locus showed higher heterogeneity, with 34 different types, ranging from 7 to 48 repeats. MLVA types that presented more than 30 repeats in P146 were found in Spain and Brazil, while shorter repeats were observed in the USA and Mexico. This simplified MLVA method proved to be an efficient tool for typing M. hyopneumoniae with a high degree of stability, repeatability, and discriminatory power. In conclusion, M. hyopneumoniae showed a high variable number tandem repeat heterogeneity and this assay can be applied in molecular epidemiology investigations within farms and productions systems.

Keywords: Adhesins VNTR Molecular typing Multiple locus variable tandem analysis (MLVA) Enzootic pneumonia

1 Introduction

Mycoplasma hyopneumoniae (M. hyopneumoniae) is the etiological agent of enzootic pneumonia, an important respiratory disease affecting pigs (Mare and Switzer, 1965; Goodwin et al., 1965). M. hyopneumoniae infections have been reported worldwide, and have an important economic impact in swine production, reducing growth rate and feed efficiency (Maes et al., 2008. Various reports have suggested the circulation of genetically diverse strains of M. hyopneumoniae in swine, as detected by different molecular techniques (Frey et al., 1992; Kokotovic et al., 1999; Vicca et al., 2003; Dubosson et al., 2004; Stakenborg et al., 2005; Stakenborg et al., 2006; Mayor et al., 2008; Nathues et al., 2011; Vranckx et al., 2011; Charlebois et al., 2014). Multiple locus variable number tandem repeat analysis (MLVA) has been used to genotype several species of bacteria, including Mycoplasma species. The use of MLVA has improved the understanding of bacterial epidemiology as it presents a high discriminatory power and can be a useful tool in outbreaks and epidemiological investigations. Other advantages include the ability to use genetic material without the need to grow the bacterium, to be performed directly from clinical specimens, and to be used as an initial high throughput screening and typing method. The fact that Mycoplasma genomes contain numerous repetitive regions within their DNA and that they are known to be active sites for genetic recombination (Rocha et al., 2002) has prompted the hypothesis that MLVA is an appropriate technique to investigate genetic variation in M. hyopneumoniae. Previously, two different MLVA assays have been developed for typing M. hyopneumoniae (Vranckx et al., 2011; Charlebois et al., 2014). However, little is known about M. hyopneumoniae heterogeneity in United States of America (USA), Brazil, Mexico, Spain, and other countries. In this study, we modified, standardized and evaluated the stability and reproducibility of a MLVA typing method

(Vranckx et al., 2011) to analyze the genetic variability of M. hyopneumoniae in samples obtained from North American, Brazilian, Mexican and Spanish herds.

2 Material and methods

2.1 M. hyopneumoniae strains

A reference strain of M. hyopneumoniae (ATCC-25095), originally isolated from a swine with enzootic pneumonia, was cultured in Friis broth (modified from Friis, 1975; no glucose solution and thallium acetate added), passaged 12 times and used as a positive control for in vitro stability and reproducibility tests. The strain was propagated for 72-168 h, at 37 ^{o}C and 400µl of each suspension were inoculated into 2 ml of fresh broth for each passage.

2.2 Previous experimental infection study

Extracted DNA was obtained from bronchial swabs collected from pigs experimentally infected with M. hyopneumoniae strain 232 (Iowa State University) via intratracheal. Briefly, bronchial swabs were collected from pigs 28 days post infection (DPI). Pigs were either born to vaccinated or unvaccinated gilts, and the piglets were vaccinated with Respisure ONE® (former Pfizer Animal Health, now Zoetis, New Jersey, USA) or remained unvaccinated. These set of samples were used to test the stability and reproducibility (Vergnaud and Pourcel, 2009) of each locus after in vivo passage in pigs.

2.3 Clinical specimens

Samples were selected based on a positive result for M. hyopneumoniae by PCR at the different institutions where they were submitted (Table 1).

2.4 DNA extraction

Purified, genomic DNA was obtained from the M. hyopneumoniae reference strain (passages 3, 6, 9 and 12) using the DNeasy Blood & Tissue Kit (Qiagen, California, United States) according to the manufacturer's instructions. The DNA from USA and Mexican samples was extracted using MagMAX™ Total Nucleic Acid Isolation Kit (Applied Biosystems, California, United States). DNA samples from Brazil (n=95) and Spain (n=25) embedded in FTA cards were recovered using the following protocol: Three punches (6 mm) from the FTA card were placed in a 1.5 ml sterile microtube, washed 2X with 200 µl of FTA Wash solution (Sigma-Aldrich, Missouri, United States) with a 5 min incubation period and after each wash the liquid was discarded. This step was followed by two washes with 200 µl of TE buffer (10 mM Tris pH 8.0, 1 mM EDTA), with a 5 min incubation period, and discarding the liquid after the washes. Thirty five µl of alkaline incubation buffer (0.1 N NaOH, 0.3 mM EDTA, pH 13.0) were added, and the samples were incubated 65°C for 5 min. Sixty five µl of neutralizing solution (0.1 M Tris-HCl, pH 7.0) were added and the samples were vortexed 5X, followed by 10 min incubation time at room temperature. Punches were removed and the maximum amount of elute was recovered by pressing the punches against the tube wall. A speed vacuum step was performed to concentrate the samples. The final step, drop dialysis, was performed by adding 15 µl of DNA to a membrane filter of 0.025µm (Millipore, Massachusetts, United States) and incubated for 25 min in a Petri dish with 20 ml of molecular grade water (BioExpress, Pennsylvania, United States). The DNA was recovered and stored at -20^{o}C until further use.

2.5 Multilple-Locus Variable number tandem repeat Analysis (MLVA)

Primers of two VNTR loci: P97 RR1 (mhp138) and P146 RR3 (mhp 684) previously described by Vranckx et al., (2011) were used in this study. The PCR reactions were performed in a final volume of 25 µl, containing 1X Qiagen Multiplex PCR Master Mix (Qiagen, California, United States), 0.2 µM of each primer (Applied Biosystems, California, United States) and 2 µl of template DNA. PCR reactions were carried out in an Eppendorf thermal cycler (Eppendorf, Hamburg, Germany) using the amplification conditions described by Vranckx et al., (2011). PCR products were analyzed by electrophoresis using 1.5% agarose gels, ran at 120 Volts for 30 min and visualized using a gel documentation system, to confirm product amplification prior to capillary electrophoresis. Amplification products were diluted 1:32 in molecular grade water and submitted to capillary electrophoresis (AB 3730xl DNA Analyzer) using GeneScan 1200 LIZ as size standard (Applied Biosystems, California, United States).

2.6 Locus stability and reproducibility tests

A reference strain of M. hyopneumoniae (ATCC-25095) was chosen to test the in vitro stability of the loci used in this study. The strain was passaged 12 times in modified Friis broth, to determine the stability of each locus. In vivo stability was evaluated by typing the DNA obtained from experimentally infected pigs with M. hyopneumoniae and confirming that the MLVA type of the strain prior to inoculation corresponded to the same type after the in vivo passage. The reproducibility of the MLVA method was tested by analyzing the DNA obtained from the reference strain and submitted more than 20 different times to MLVA analysis.

2.7 Data analysis

Data files obtained as a result of capillary electrophoresis (.fsa) were imported into Bionumerics version 7.0 (Applied Maths, East Flanders, Belgium) to determine fragment size and to estimate the number of repeats in the amplicons. Each sample was estimated a number based on the size of the highest peak (bp count; highest fragment observed in the electropherogram was chosen for analysis, discarding artifacts). The offset was calculated based on the sum of the forward and the reverse offset, and adjusted taking into account the difference between the theoretical fragment size and the observed fragment size. For P97, an offset of 206 bp was used, and for P146 the offset was 201 bp. A dendrogram was constructed using unweighted pair group method with arithmetic means (UPGMA) based on the categorical values of the number repeats, based on the method ability to construct a dendrogram by successive clustering, thus increasing the robustness of the dendrogram. The Simpson's index of diversity of combined VNTR loci was calculated by using the Hunter-Gaston diversity index – HDGI (Hunter and Gaston, 1988). The discriminatory index is the measure of the probability of diversity which takes into account the number of types present, as well as the relative abundance of each type. Discriminatory indexes were calculated for each country and for all samples combined. To be considered ideal, the discriminatory index should be on the other of 0.95 (Van Belkum et al., 2007).

3 Results

The assay standardized in this investigation was modified and evaluated using two loci, namely P97 RR1 and P146 RR3. The peaks for P97 and P146 were identified on electropherograms by molecular size ranges and numbers of repeats were then converted to

categorical values based on the number of repeats. A composite of the number of repeats for both loci was used to define a MLVA type. Each MLVA type consists of 2 numbers: a) number of repeats in RR1 P97, and b) number of repeats in RR3 P146, divided by a dash.

3.1 Locus stability and reproducibility tests

The stability of the repeats in the MLVA assay were confirmed in vitro after analysis of 12 passages of a M. hyopneumoniae strain, and in vivo by testing the DNA obtained from a previous study in which pigs were experimentally infected with M. hyopneumoniae. In both tests the MLVA type in the sample returned the same MLVA type (i.e. 14-21) as the parental reference strain or the strain used for experimental infection, even in pigs previously vaccinated or born to vaccinated dams (results not shown). The reproducibility of the method was confirmed after analysis of the DNA obtained from the reference strain which was tested over 20 times, in different runs. The MLVA type was identified as the same at all times it was tested (data not shown).

3.2 Discriminatory power

The Simpson's diversity index for the assay was calculated for each country and in combination for the four countries. The index of diversity was D= 0.979 for USA (n=209), D= 0.880 for Mexico (n=26), D= 0.865 for Brazil (n=95), D = 0.99 for Spain (n=25), and the combination of the discriminatory power for all samples (n=355) included in this study was D=0.976.

3.3 MLVA typing

3.3.1 USA samples

The genetic variability of M. hyopneumoniae was investigated in 209 clinically specimens submitted to the UMN-VDL from geographically distinct US farms. Based on these results, analysis of the combination of the 2 loci (P97 and P146) revealed 87 MLVA types for all USA samples in the study (Figure 1). The most frequent MLVA types were: 9-26 (7.2%), 15-25 (6.2%), 15-21 (5.2%), 11-15 (4.7%), and 14-21(4.3%). In the state of Minnesota 54 MLVA types were identified (n=107 samples). The most frequent types were 15-23 (12.2%), 15-21 (10.3%), and 8-7 (6.5%). However, no grouped population structure was observed on the basis of geographical location.

3.3.2 Mexican samples

The clinical specimens originated from Mexico presented 12 different MLVA types (Figure 2A). The most frequent MLVA type identified was 10-18 (30.7%) and was obtained from one production system. Three samples originated from a second production system in Mexico showed only one type, namely 10-17.

3.3.3 Brazilian samples

In Brazil, 39 different MLVA types were identified (Figure 3). The most frequent type observed was 12-14 (35.8%) which was identified in 8 different states and the MLVA type 10-18 (6.3%), which was found only in one state.

3.3.4 Spanish samples

Spanish samples presented 22 different types (Figure 2B). The type 8-21 was identified in two strains isolated in 2006 and 2010. Other two types, 8-32 and 8-35, also were identified in two strains each.

4 Discussion

In the study presented here, we investigated the genetic variability of M. hyopneumoniae in specimens from clinically relevant cases submitted from geographically distinct locations in the USA, Brazil, Mexico, and Spain. To address this issue we standardized a simple and high resolution MLVA test. The assay proved to be an efficient tool for typing M. hyopneumoniae which can be standardized in diagnostic laboratories as it offers high stability and reproducibility. However, the fact that the equipment to perform the capillary electrophoresis and the hardware to analyze the results are costly, should be taken into account submit the samples to facilities that own this apparatus. Under the conditions of this study, a high number of M. hyopneumoniae MLVA types were identified within and between countries, and no common MLVA type was observed in samples from all the regions included in this investigation.

In the USA, samples were obtained from different regions, including different states. Comparison among states demonstrated that states with less than 7 samples revealed 1-4 MLVA types. However, the limited and not comparable sample size among states has an effect on the number of MLVA types found in each state. Mexican samples were related to two different production systems, with one production system presenting only one MLVA type and the other production system presenting 11 different MLVA types found in 15 different farms. The limited number of samples originated from Mexico could have influenced the observation of these two

clusters. In Brazil samples were originated from a diagnostic laboratory that receives clinical specimens from various states and one frequent type was found in 8 different states. Furthermore, it was possible to observe heterogeneity of M. hyopneumoniae within state, in all states analyzed. In Spain the samples were related to the main pig production areas, located in Catalonia and Aragon, which can explain the higher heterogeneity found in that country. Samples that were obtained from diagnostic laboratories (Brazil, Mexico, and USA) showed higher heterogeneity in the P97 gene when compared to the isolates originated from Spain. Spanish samples had limited variation in the P97 locus, with only five types. The P146 locus had demonstrated higher variation in both set of samples, strains and clinical specimens. In total, 17 different types were observed in the P97 locus, and these types ranged from 2 to 18 repeats. On the other hand, P146 presented 34 different types ranging from 7 to 48 repeats. The types that presented more than 30 repeats in P146 were found only in Spain and Brazil. This observation is in agreement with reports from De Castro et al., 2006, in which Brazilian samples ranged from 18 to 44 repeats in the same repeat region. The different methods performed to extract the DNA could be biased the comparison between the countries. Nevertheless, no relationship has been demonstrated between number of repeats and bacterial virulence.

In the results presented by Vranckx et al., 2011 and Charlebois et al., 2014 the MLVA types for each sample were not disclosed. Therefore, it was not possible to compare the number of repeats found in our study with those previously published. The large number of MLVA types found in this investigation suggests a large diversity in M. hyopneumoniae circulating in the swine herds. Also, evidence of multiple M. hyopneumoniae variants was observed in single samples (13.8%; 49/355), which was observed by the amplification of more than one peak in the capillary electrophoresis analysis. These results suggest infection with more than one strain in

the same pig, as previously suggested (Vranckx et al., 2011; Charlebois et al., 2014). The high numbers of M. hyopneumoniae variants identified in this investigation are in agreement with other reports which identified clonal variants in the single herds and in different herds (Nathues et al., 2011; Vranckx et al., 2012; Charlebois et al., 2014). Clonal variants present in the same herd showed a low variability among them, and shared the same number of repeats in one locus and variable number of repeats in the other locus. In large production systems, a different pattern of clonality was observed which is in agreement with the results of Vranckx et al., 2012.

The M. hyopneumoniae reference strain used to standardize this MLVA is the parental strain of US232, a reference strain in the USA (Minion et al., 2004). Both samples showed the same type (14-21) when evaluated with this method. This type was only identified in the USA, which could be explained by the origin of the isolate. There was not a common MLVA type across all countries. However, some types appeared to be shared among countries.

5 Conclusion

The MLVA typing method developed in this study is an improved assay for differentiation of M. hyopneumoniae variants in clinical specimens. This assay revealed a high discriminatory index, suggestive of high diversity of M. hyopneumoniae in the investigated herds, indicating that multiple M. hyopneumoniae variants are circulating in swine herds in the USA, Brazil, Mexico and Spain. Further analysis of samples collected longitudinally from diverse geographic locations and clinical presentation is necessary to investigate if a nonrandom distribution of genotypes is present among strains.

Conflict of Interests

The authors of this manuscript declare they have no competing interest.

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Figures

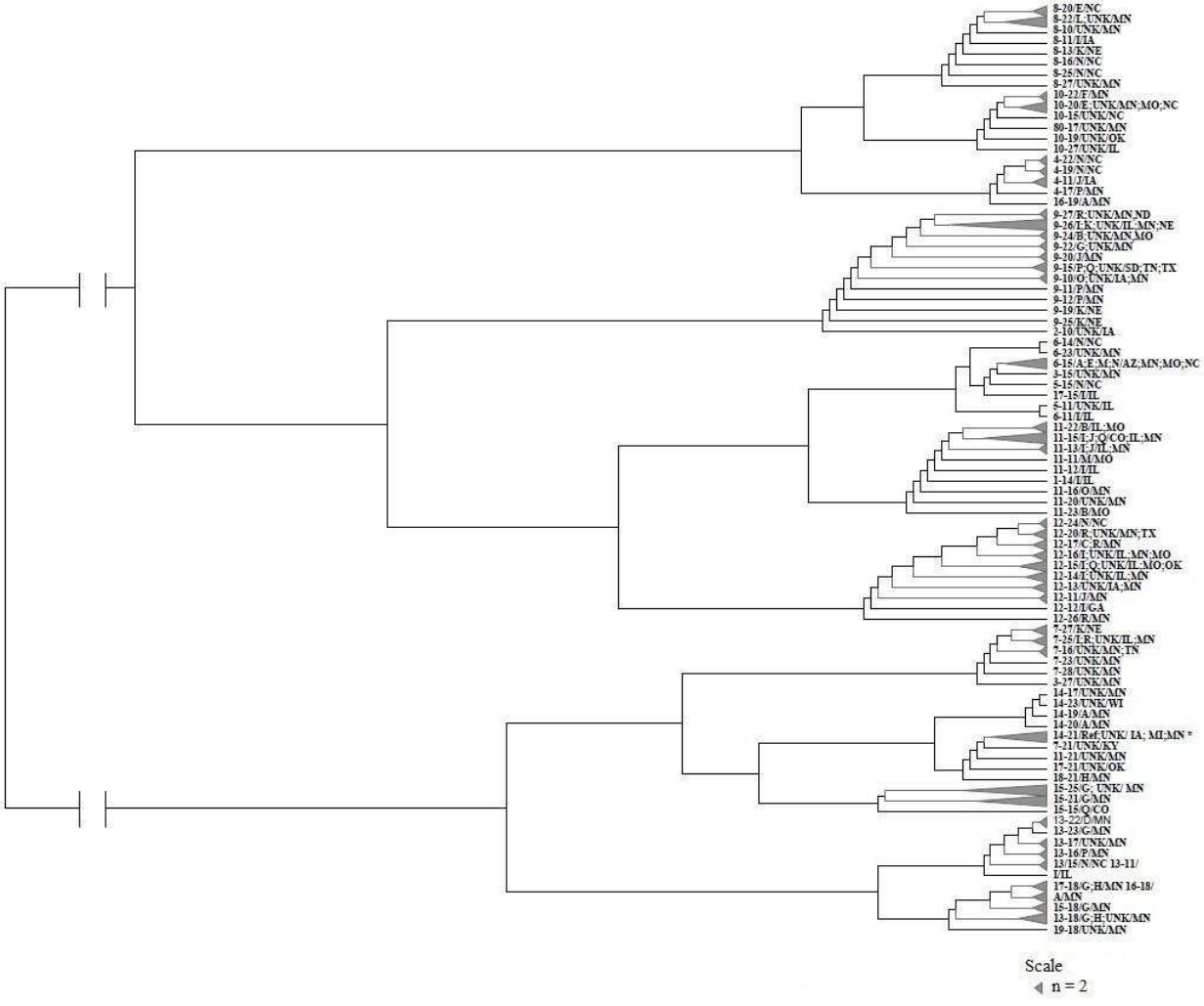
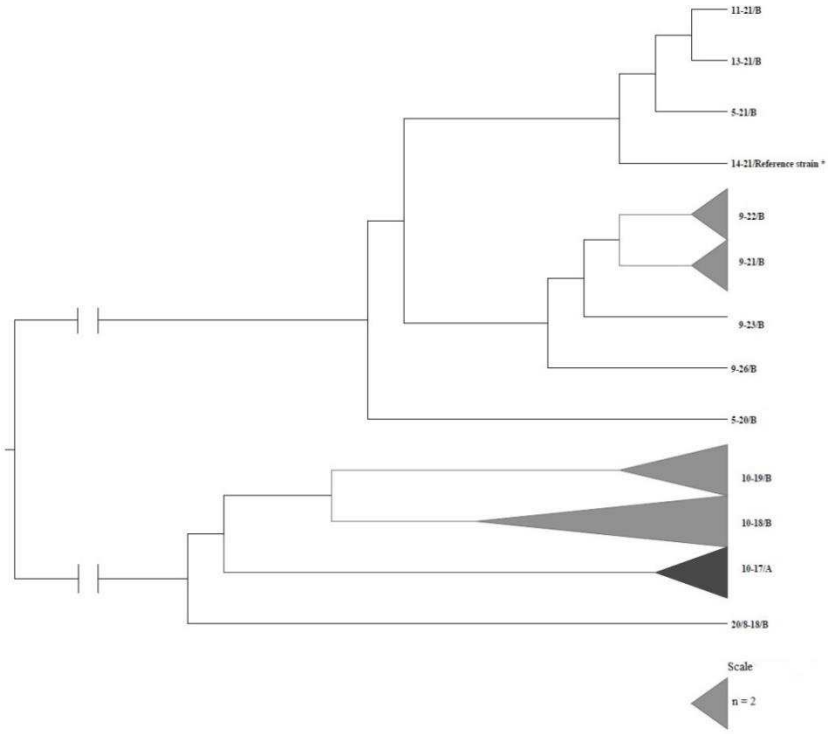
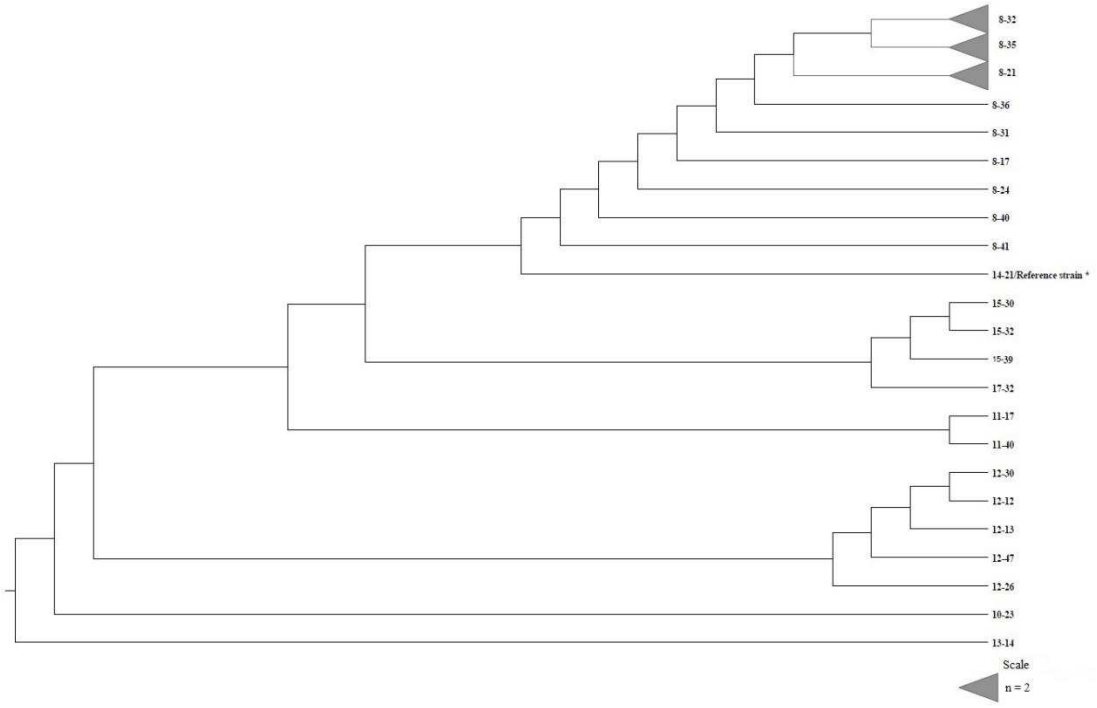


Figure 1 - Dendrogram showing the distribution of M. hyopneumoniae MLVA types in clinical specimens in the USA. The MLVA type is indicated by the number of repeats in each locus (e.g. 14-21), followed by the production system where the type was identified (A to R, UNK = unknown) and the state where the type came from (USA state code). The size of the gray triangle represents the frequency of the type. The MLVA type with asterisk represents the reference strain (ATCC-25095). The dendrogram was generated using the unweighted pair group method with arithmetic means (UPGMA) and cut to fit in the page.

A


11-21/B
13-21/B
5-21/B
14-21/Reference strain *
9-22/B
9-21/B
9-23/B
9-26/B
5-20/B
10-19/B
10-18/B
10-17/A
20/8-18/B
Scale
n = 2


8-32
8-35
8-21
8-36
8-31
8-17
8-24
8-40
8-41
14-21/Reference strain *
15-30
15-32
15-39
17-32
11-17
11-40
12-30
12-12
12-13
12-47
12-26
10-23
13-14
Scale
n = 2

B

Figure 2 - Dendrogram showing the distribution of M. hyopneumoniae MLVA types in clinical specimens in A) Mexico and B) Spain. A) The MLVA type is indicated by the number of repeats in each locus (e.g. 14-21), followed by the production system in which the sample was identified (A and B). The black triangle represents the production system A and the gray triangles the productions system B. B) The size of the gray triangle represents the frequency of the referred type. Dendrograms were generated using UPGMA.

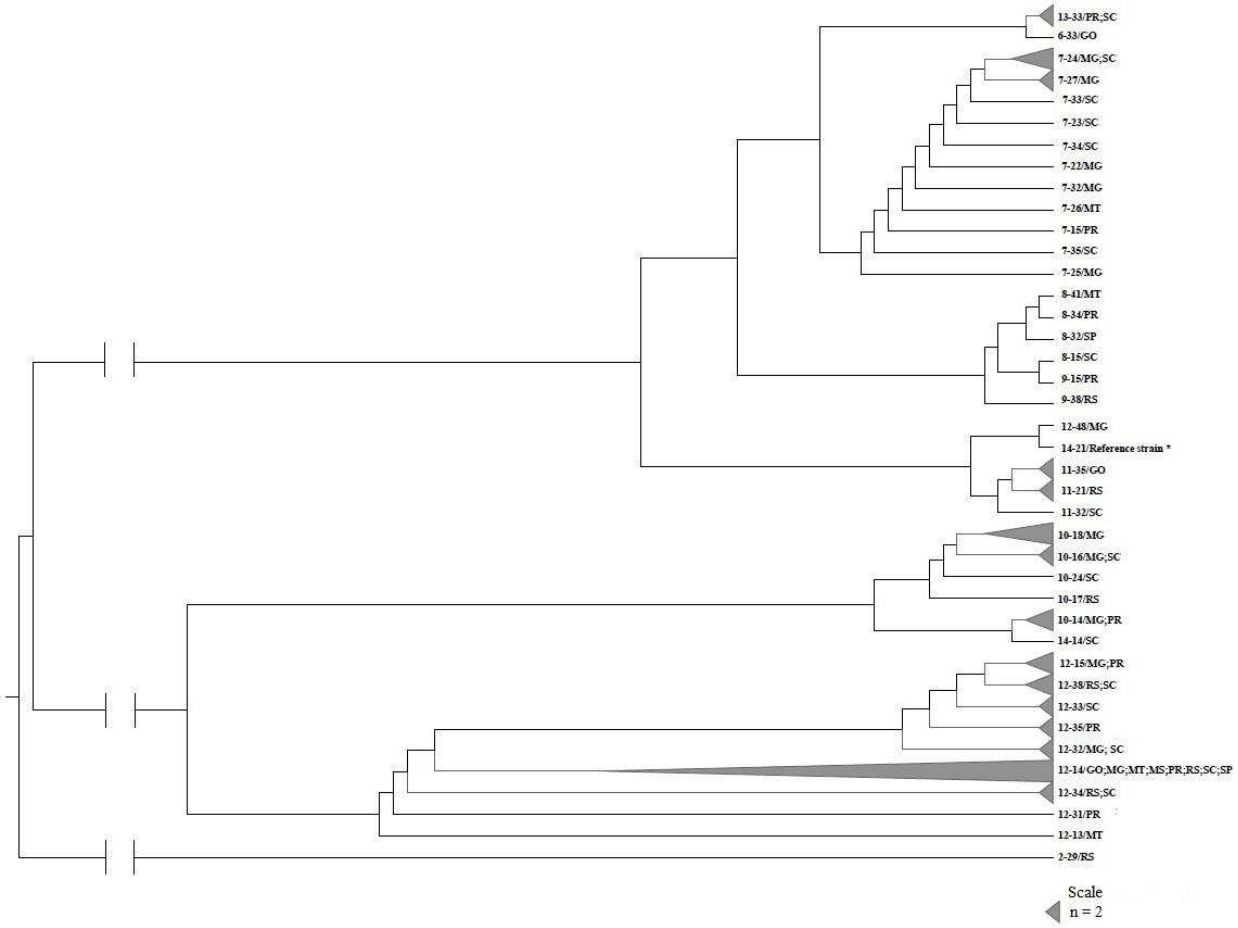


Figure 3 - Dendrogram of the distribution of M. hyopneumoniae MLVA types in clinical specimens in Brazil. The MLVA type is indicated by the number of repeats in each locus (e.g. 14-21), followed by state in which the samples were obtained (Brazil two letter state code). The

size of the gray triangle represents the frequency of the referred type. The MLVA type with asterisk represents the reference strain used in the analysis (ATCC-25095). The dendrogram was generated using UPGMA and cut to fit in the page.

Table

Table 1. Clinical specimens processed for M. hyopneumoniae MLVA typing.

Country	N° of samples [4]	Type of samples	Time period of sample collection	Number of units from which samples originated			Sample matrix	DNA extraction method	PCR assay used to detect sample positivity
				States	Prod. Systems	Farms			
Brazil[1]	95	Bronchial swabs	March 2013 – March 2014	8	43	76	FTA card	FTA card protocol[5]	Stakenborg et al., 2006
Mexico[2]	26	Bronchial swabs	June-November 2012	2	2	16	Extracted DNA	MagMAX™ Total Nucleic Acid Isolation Kit[6]	VetMAX™ M. hyopneumoniae[6]
Spain[3]	25	Bacterial isolates	2005, 2006, and 2010	2	24	24	FTA card	FTA card protocol[5]	Calsamiglia et al., 1999
USA[2]	209	Bronchial and nasal swabs	June-November 2012	17	82	208	Extracted DNA	MagMAX™ Total Nucleic Acid Isolation Kit[6]	VetMAX™ M. hyopneumoniae[6]

[1] Samples were submitted to Microvet, Ltda. (Veterinary Diagnostic Laboratory in Viçosa, MG, Brazil).

[2] Samples submitted to the Veterinary Diagnostic Laboratory at the University of Minnesota (St. Paul, MN, USA).

[3] Bacterial isolates were obtained from Centre de Recerca en Sanitat Animal, Universitat Autonoma de Barcelona (Bellaterra, Catalonia, Spain).

[4] Samples from Brazil, Mexico and USA were submitted to the diagnostic laboratories based on the suspicion of infection with M. hyopneumoniae. No information available regarding vaccination or antibiotic treatment(s). Specific sample information has been omitted in order to protect client data.

[5] Described in the Materials and methods section.

[6] (Applied Biosystems, California, USA).

CHAPTER II - Genotyping of Mycoplasma hyorhinis using multiple-locus variable number tandem repeat analysis

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Title Page

Genotyping of Mycoplasma hyorhinis using multiple-locus variable number tandem repeat analysis

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Abstract

Mycoplasma hyorhinis (M. hyorhinis) has re-emerged as an important swine pathogen in recent years causing significant economic losses in post weaning pigs. Genetic variability of M. hyorhinis has been described based on different molecular methods that have limited resolution and reproducibility. The present study was undertaken to develop a molecular epidemiological typing tool for M. hyorhinis based on multiple loci of variable number of tandem repeats in its genome, termed MLVA. The typing method was designed on the basis of the number of repeats in two hypothetical proteins, MHR_0152 and MHR_0298. A total of 205 samples were analyzed, including field isolates, clinical specimens, and a reference strain. Analysis of the combination of the 2 loci revealed 16 MLVA types in 165 of the 205 samples. In the remaining forty samples only one locus could be amplified. The most frequent types obtained from the set of samples were 8-4 (36.9%), 8-3 (11.5%), 7-4 (11.5%), 9-4 (10.9%) and 10-4 (9.3%). The Simpson's diversity index for the assay was D= 0.814 when the 165 samples were taken into account. No clustering was observed based on the geographical location, sample type, or year of isolation or sampling. The MLVA assay developed in this investigation showed to be a reproducible and portable assay which could be easily performed and transferred to other laboratories. The use of this technique will assist in epidemiological investigations and can be used to improve the understanding the molecular biology of M. hyorhinis variants.

Key words: Mycoplasma hyorhinis, VNTR, MLVA, genotyping, polyserositis, swine

1 Introduction

Mycoplasma hyorhinis is commonly present in the respiratory tract of pigs (Switzer, 1955). M. hyorhinis can be present in normal lungs, but it is more frequently found in pneumonic lungs, where it is considered a secondary pathogen (Kobayashi et al., 1996; Kawashima et al., 1996; Lin et al., 2006). M. hyorhinis can cause polyserositis and arthritis in animals from 3 to 10 weeks of age when it becomes systemic (Potgieter and Ross, 1972; Friis and Feestra, 1994). An increase in the detection of the M. hyorhinis as an etiological agent of polyserositis and arthritis in piglets in recent years demonstrates the relevance of this pathogen in the swine industry as an emerging challenge (Rovira et al., 2010).

When compared to other mycoplasmas, M. hyorhinis exhibits an aggressive growth due to differences found in components involved in metabolism, which is suggestive of evasion of the host immune system (Siqueira et al., 2013). Population diversity within the M. hyorhinis species is due in part to the occurrence of variation in the expression of surface lipoproteins (Rosengarten and Wise, 1991). It has been suggested that the source of genetic variation in the variable lipoproteins (Vlp) is the insertion and deletion of tandem repeats (VNTR) within the 3'region in the Vlp genes (Rosengarten and Wise, 1991; Yogev et al., 1991; Citti et al., 2000).

The heterogeneity of M. hyorhinis has been the focus of a limited number of studies in the past years. Restriction endonuclease analysis was applied by Darai et al. (1982) in a strain collection of M. hyorhinis to verify the characteristic of fragment pattern of DNA bands. More recently, two typing methods were used to identify the genetic diversity in M. hyorhinis, namely sequencing of the p37 gene and a multilocus sequencing typing (MLST; Tocqueville et al., 2014). Among the bacterial genotyping methods, multiple-locus variable-number tandem-repeat analysis (MLVA) has been used to type diverse Mycoplasmas (McAuliffe et al., 2007; Ma et al.,

2008; Degrange et al., 2009; Manso-Silván et al., 2011; Pinho et al., 2012; Vranckx et al., 2011; Charlebois et al., 2014), but has not been applied to type M. hyorhinis. Therefore, the present study was performed with the objective of developing a MLVA assay based on VNTRs specific for M. hyorhinis.

2 Materials and methods

2.1 Bacterial strains and culturing conditions

A total of 116 M. hyorhinis strains were used in this study, including a reference strain (ATCC 17981). The 115 field isolates were obtained from clinical cases from diseased pigs submitted to the Veterinary Diagnostic Laboratory of the University of Minnesota (UMN-VDL) during 2010-2012. The isolates originated from 10 U.S. States (n=112) and Mexico (n=3). In twelve cases more than one sample per pig was tested. Isolates were cultured from different sites within the pig, i.e. pleura, pericardium, joint, peritoneum, nasal cavity, and lung. Isolates were propagated in modified Hayflick's medium (Kobisch and Friis, 1996) for 7-14 days at 37^{o}C and confirmed by species specific real time PCR (Clavijo et al., 2014).

2.2 Clinical specimens

Eighty-nine clinical samples including bronchial swabs, pleural and pericardial swabs, submitted to the UMN-VDL in February – March of 2014 were selected for this study. Samples were selected based on the positivity to M. hyorhinis by species specific real-time PCR developed at the University of Minnesota, Veterinary Diagnostic Laboratory.

2.3 In vitro stability and reproducibility tests

Five M. hyorhinis isolates, including the reference strain (ATCC 17981), were chosen for use in the stability testing. Each strain was passaged 10 times in modified Hayflick's medium to determine the stability of each locus in the MLVA analysis. The reproducibility of the assay was tested by analyzing 10 different MLVA runs of the DNA obtained from the first passage of the reference strain.

2.4 DNA extraction

Purified genomic DNA was obtained from the 116 M. hyorhinis isolates and the 10 passages from five isolates employed in the stability test using the DNeasy Blood & Tissue Kit (Qiagen, California, United States of America) according to the manufacturer's instructions. The MagMAX™ Total Nucleic Acid Isolation Kit (Applied Biosystems, California, United States of America) was used to extract the DNA from clinical specimens obtained from UMN VDL (n=89).

2.5 Identification of VNTRs

The variable number tandem repeat markers were identified in the sequenced genome of M. hyorhinis HUB-1 (NC_014448) by using the Tandem Repeat Finder software (Benson, 1999). Loci were chosen on the basis of copy number of repeats in each locus. The presence of at least four repeats of less than 40 bp was the criteria for the locus to be included in this study. A set of 6 VNTRs that fit the selected inclusion criteria was identified. Once the VNTR sequences were chosen, conserved primers were designed in the flanking regions of the tandem repeat using Primer3 software, available online (Rozen, 1998). The selected VNTRs were tested with specific

primers to amplify DNA from a subset of 20 M. hyorhinis isolates and the reference strain as a preliminary selection. Each locus was amplified individually by PCR and was analyzed by conventional agarose gel electrophoresis. The PCR products were purified using a QIAquick PCR Purification Kit (Qiagen) and were sequenced to confirm the presence of tandem repeats and to determine the number of repeats in each locus. By this approach, two VNTR loci, which corresponded to two hypothetical proteins MHR_0152 (accession number YP_003856162) and MHR_0298 (accession number ADM21765), were selected for analysis of field samples.

2.5 PCR amplification for MLVA

The two loci selected for MLVA (Table 1) were multiplexed and the amplifications were performed in an Eppendorf thermal cycler (Eppendorf, Hamburg, Germany) in a final volume of 25 µl. The reaction mixture contained 1X Qiagen Multiplex PCR master Mix (Qiagen), 0.2 µM of MHR_0152 primers, 0.6 µM of MHR_0298 primers (primer concentration ratio resulted from an optimization test (data not showed); Table 1; Applied Biosystems), 1.5 mM $MgCl_2$, and 2 µl of template DNA. PCR conditions were 95°C for 15 min, followed by 35 cycles of 94°C for 30 s, 50°C for 45 s, and 72°C for 1 min and with a final extension of 72°C for 10 min. PCR products were analyzed by electrophoresis using 1.5% agarose gels, ran at 120 Volts for 40 min and visualized using a gel documentation system. Amplification products were diluted 1:32 in molecular grade water (BioExpress, Pennsylvania, United States of America) and submitted for capillary electrophoresis using GeneScan 1200 LIZ as size standard (Applied Biosystems).

2.6 Data analysis

To determine the number of repeats in each locus, files containing the capillary electrophoresis results were imported into Bionumerics version 7.0 (Applied Maths, East Flanders, Belgium). A dendrogram was generated by using unweighted pair group method with arithmetic means (UPGMA) based on the categorical values of the number of repeats. The algorithm was selected due to its ability to construct a dendrogram by successive clustering using an average-linkage method. Since the algorithm considers that pairwise distances contribute equal to clustering the data, it automatically clusters variations in one or the other repeats. For example all "x"- 4's are clustered in one clade while all "y"- 3's are clustered together. The Simpson's index of diversity (HGDI; Hunter and Gaston, 1988) was estimated to calculate the diversity of the combined VNTR loci.

3 Results

3.1 Identification of VNTRs

Preliminary analysis of six loci, namely vlpA, vlpB, vlpC, vlpE, MHR_0152 and MHR_0298 from a M. hyorhinis reference strain (ATCC 17981) from 20 isolates showed that only two loci were polymorphic and repeatable. The other four loci failed to amplify the DNA in the isolates and therefore were eliminated from this study. The assay developed in this study was standardized targeting the number of repeats present in the loci MHR_0152 and MHR_0298. Sequencing of the PCR products of the two loci from each of the 20 isolates and the reference strain confirmed the sizes and sequences of the individual VNTR loci.

3.2 In vitro stability and reproducibility tests

The stability of the two VNTR markers selected for this assay was studied in five isolates after 10 passages in Modified Hayflick's medium. Analysis of the five strains resulted in identical MLVA profiles for both markers. The MLVA type of the reference strain was identified as the same in all repeats tested, confirming the reproducibility of the method (data not shown). The use of the stability and reproducibility test were important to perform the validation of the method. Lack of in vivo tests may not guarantee to state that there is stability in the locus used in this approach, since the M. hyorhinis could be under selection pressures and do genetic variation, especially in the Vlp genes.

3.3 MLVA typing

The genetic variability of M. hyorhinis was investigated in 205 samples, including a reference strain, 115 isolates, and 89 clinical specimens submitted to the UMN-VDL from geographically distinct U.S. regions (13 States) and Mexico. Based on these results, analysis of the combination of the 2 loci (MHR_0152 and MHR_0298) revealed 16 MLVA types for all samples tested in the study (Figure 1). The MLVA type is the result of combining the number of repeats for both loci, number of repeats in MHR_0152 followed by the number of repeats in MHR_0298, divided by a dash. A total of 38 out of 89 clinical specimens, and 2 out of 115 isolates only amplified for one locus (MHR_0298). Thus, a total of 165 samples were typed by this method. The most frequent types were: 8-4 (36.9%), 8-3 (11.5%), 7-4 (11.5%), 9-4 (10.9%) and 10-4 (9.3%). The Simpson's diversity index for the assay was D= 0.814 when the 165 samples were taken into account. In total, 9 different types were observed in the MHR_0152 locus, and these types ranged from 6 to 19 repeats. In contrast, MHR_0298 presented only 3

different types ranging from 3 to 5 repeats. No clustering was observed based on the geographical location, sample type or year of isolation (Table 2). In the 12 cases where more than one sample per pig was analyzed (different sample types), four pigs showed an identical MLVA type in all samples tested (Table 3), while in the other eight cases, the MLVA type differed based on the sample type tested (Table 3).

4 Discussion

The issue addressed in the present study was the heterogeneity of M. hyorhinis using a molecular tool based on the VNTRs present in the genome of this bacterium. An MLVA assay was developed, which was reproducible and easy to interpret. A limited number of M. hyorhinis MLVA types were identified in the set of isolates and clinical specimens used in this study. The heterogeneity of M. hyorhinis, observed in this investigation is in agreement with the results reported by Tocqueville et al. (2014).

MLVA is a commonly used method for typing mycoplasmas and uses the variation in the number of VNTRs to differentiate samples. M. hyorhinis repeat motifs are located mainly in the vlp genes, however the low G+C content of these regions limited the area for primer design. The choice of two hypothetical proteins as target loci in this investigation allowed for the development of a simple, reproducible and portable assay.

In this study, we described 16 MLVA types. This finding is consistent with the diversity data reported by others. The results from a restriction endonuclease study using three different enzymes BstEII, XhoI and SacI demonstrated variability in 6 M. hyorhinis strains (Darai et al., 1982). Tocqueville et al. (2014) using the sequencing of the p37 gene, found 18 distinct nucleotides sequences among 33 strains. And when the MLST scheme was evaluated, the same

author reported 29 different MLST types among the same 33 strains evidencing a high variability in this organism.

The MLVA method developed in this investigation was used to study the frequency distribution of types in a set of isolates and clinical specimens. The number of repeats in each locus showed limited variation however, genetic variation of strains could still be identified. In this study, the association between isolates or clinical specimens originating from polyserositis sites and MLVA type was not observed, which is in disagreement with a previous study (Tocqueville et al., 2014). However, it is important to note that the MLST tool developed by Tocqueville et al. (2014) targets housekeeping genes, which are conserved genes different from the highly variable VNTRs used as a target in this study.

Analysis of results suggest that one strain with the a unique MLVA type was involved in the infectious process in cases where the same MLVA type was found in different sample types, in the same animal (Table 3). This was observed in 4 out of 12 cases in which various samples were analyzed from the same pig. However, in 8/12 cases multiple MLVA profiles were identified in different sample types from the same animal, which suggests that the pig was colonized with different strains. At the population level, the MLVA method was able to detect the genetic diversity of M. hyorhinis. It would be interesting, at epidemiological point, to compare the MLST profile to the MLVA type to perfome a more appropriate characterization of the strain. Due the highly discriminatory power of the MLST.

The absence of amplification of one locus in the clinical specimens was a limitation of this technique. However, it is our hypothesis that the presence of the DNA of multiple strains in the sample could have interfered with the results of the typing method, since the lack of amplification of one locus was not observed in most of the isolates. Also, the concentration of

the DNA used in the PCR reaction could have been another factor involved in the failure of amplification of one locus. It is strongly recommended to isolate the M. hyorhinis from the clinical samples, since cultivation of this Mycoplasma species is relatively easy to perform and will positively affect the success of the results.

It should be taken into account that all samples used in this investigation were obtained from sick pigs, which could bias the results. Since natural colonization was not the focus of this study, only a few isolates from nasal swabs were tested. In three cases in which nasal samples and other samples types from the same pig were analyzed, the MLVA type observed in the nasal cavity was different from the MLVA type found in other samples, which may suggest that not all the strains that colonize the nasal cavity are able to cause systemic disease. Nevertheless, the virulence of the M. hyorhinis isolate was not established in this investigation. Further studies should investigate the virulence of the M. hyorhinis.

Finally, the typing approach developed was able to identify the heterogeneity of M. hyorhinis, however the clonality of this microorganism was also confirmed in this study. The stability and reproducibility test were performed and the results obtained in both tests were used to validate the performance of the assay developed. It would be interesting to evaluate this MLVA method in outbreak investigations to confirm the level of discrimination of this typing method.

5 Conclusions

The MLVA assay developed in this investigation is a reproducible, fast, and easy to perform assay when compared to the other molecular typing methods used to type M. hyorhinis. An advantage of MLVA is that it does not require a sequencing step, which can simplify and

shorten the turnaround. The MLVA established in this study allowed obtaining information on the genetic heterogeneity of M. hyorhinis. This information will be useful for further epidemiological studies.

Conflict of Interests

The authors of this manuscript declare they have no competing interest.

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Figure

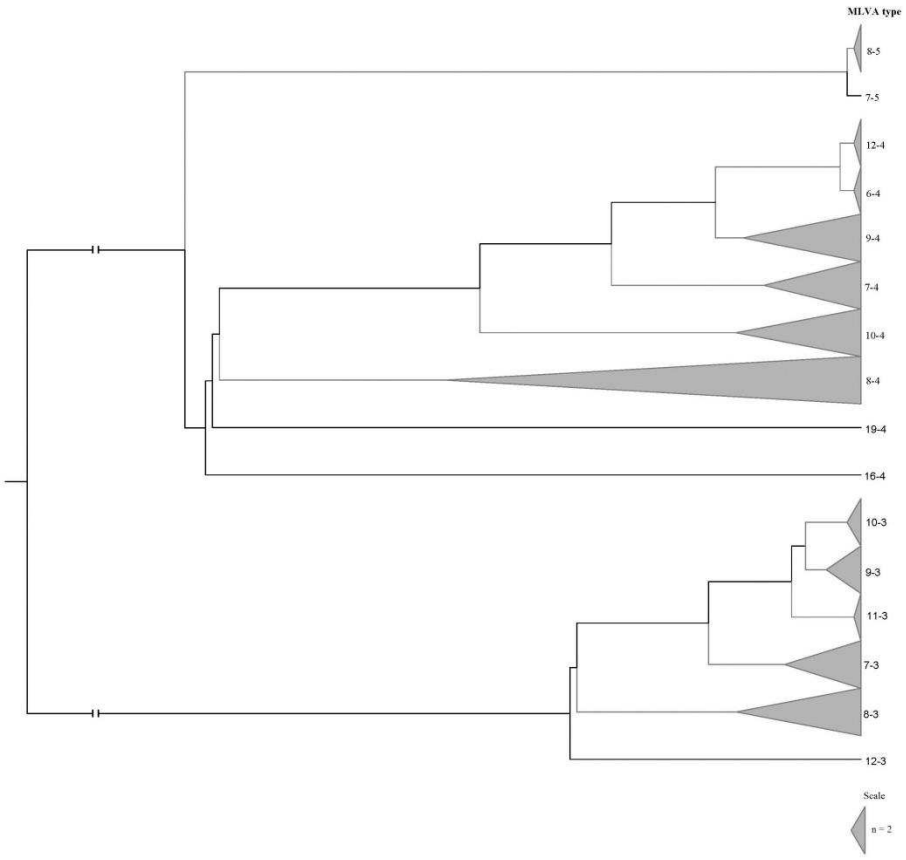

Figure 1 – Dendrogram showing the distribution of M. hyorhinis MLVA types. The MLVA type is indicated by the number of repeats in each locus for genes MHR_0152 – MHR_0298 (e.g. 8-4). The size of the gray triangle represents the frequency of the type. The dendrogram was generated using the unweighted pair group method with arithmetic means (UPGMA) and cut to fit the page. The algorithm considers that pairwise distances contribute equal to clustering the data, it automatically clusters variations in one or the other repeats. For example all "x"- 4's are clustered in one clade while all "y"- 3's are clustered together.

Tables

Table 1 - Primers used for multiplex PCR amplification for MLVA

Genes	Type	Sequence	Size of repeat
mhr 0152	Forward	**5´-6-FAM**-ACAAGAACAAGTTAATGCTGATAATCC-**3´**	3 bp-AGT
	Reverse	**5´**-TGAGAATTAGAACTAGTTTGGGTTTCTT-**3´**	
mhr 0298	Forward	**5'-VIC**-TCTTCTCAGTCTTTGATATATTGCTTG-**3'**	9 bp – TTTTTTGTA
	Reverse	**5'**-AAGTGAAGATACTCGCTTTGAAGA-**3'**	

Table 2- Characteristics of the 165 M. hyorhinis used in this study

MLVA type	Frequency		Tested material	Sample type	Geographical region	Year of isolation/ sampling
	Total	Per sample type				
6-4	2	1	Isolate	NA	NA	NA
		1	Clinical specimen	Pleura	Midwest	2014
7-3	12	2	Clinical specimen and isolate	Bronchial swab	Midwest	2011, 2014
		1	Isolate	Bursa	Midwest	2011
		4	Isolate	Joint	Midwest	2011
		1	Isolate	NA	NA	NA
		3	Isolate	Pericardium	Midwest	2011
		1	Isolate	Pleura	Midwest	2011
7-4	19	1	Isolate	Air sample	Midwest	2012
		4	Clinical specimen and isolate	Bronchial swab	Midwest and Southwest	2011, 2012, 2014
		1	Isolate	NA	NA	NA
		6	Clinical specimen and isolate	Pericardium	Midwest and Southwest	2010, 2012, 2014
		7	Clinical specimen and isolate	Pleura	Midwest and Southwest	2010, 2011, 2012, 2014
7-5	1	1	Clinical specimen	Bronchial swab	Midwest	2014
8-3	19	5	Clinical specimen and isolate	Bronchial swab	Midwest	2011, 2014
		2	Isolate	Joint	Midwest	2010, 2011
		4	Isolate	NA	NA	NA
		3	Isolate	Nasal swab	Midwest	2012
		1	Isolate	Pericardium	Midwest	2011
		1	Isolate	Peritoneal swab	Southwest	2012
		3	Clinical specimen and isolate	Pleura	Midwest	2011, 2014
8-4	61	34	Clinical specimen and isolate	Bronchial swab	Midwest and Southwest	2010, 2011, 2012, 2014
		2	Isolate	Joint	Midwest	2010, 2011
		5	Isolate	NA	NA	NA
		2	Isolate	Nasal swab	Southwest	2012
		8	Clinical specimen and	Pericardium	Midwest, Southwest and	2011, 2012, 2014

			isolate		Mexico	
		10	Clinical specimen and isolate	Pleura	Midwest, Southeast and Southwest	2010, 2011, 2014
8-5	2	1	Isolate	Joint	Midwest	2011
		1	Clinical specimen	Pleura	Midwest	2014
9-3	6	1	Isolate	Bronchial swab	Midwest	2011
		2	Isolate	Joint	Midwest	2010
		2	Isolate	NA	NA	NA
		1	Isolate	Nasal swab	Midwest	2012
9-4	18	5	Clinical specimen and isolate	Bronchial swab	Midwest and Southwest	2011, 2012, 2014
		2	Isolate	Joint	Midwest	2010
		3	Isolate	Nasal swab	Midwest and Southwest	2011, 2012
		6	Clinical specimen and isolate	Pericardium	Midwest, Southwest and Mexico	2011, 2012, 2014
		2	Clinical specimen and isolate	Pleura	Midwest	2012, 2014
10-3	3	2	Isolate	Nasal swab	Midwest	2011, 2012
		1	Isolate	Pericardium	Midwest	2012
10-4	15	6	Clinical specimen and isolate	Bronchial swab	Midwest	2011, 2012, 2014
		1	Isolate	Joint	Midwest	2011
		4	Isolate *	Nasal swab	Midwest and Southwest	2011, 2012
		3	Isolate	Pericardium	Midwest and Mexico	2010, 2011
		1	Isolate	Pleura	Southwest	2012
11-3	2	2	Isolate	Bronchial swab	Midwest	2011
12-3	1	1	Isolate	NA	NA	NA
12-4	2	1	Isolate	NA	NA	NA
		1	Isolate	Pleura	Northeast	2011
16-4	1	1	Isolate	Pleura	Southeast	2010
19-4	1	1	Clinical specimen	Pleura	Midwest	2014

* Reference strain - ATCC 17981

Table 3 – M. hyorhinis MLVA types identified in pigs from which more than one sample type was evaluated.

Sample number	Sample type	MLVA type
1	Bronchial swab	10-4
	Joint	10-4
2	Nasal swab	9-4
	Bronchial swab	9-4
3	Bronchial swab	8-4
	Pericardial swab	8-4
4	Pericardial swab	7-3
	Joint	7-3
	Pleura	7-3
	Bursa	7-3
5	Pleura	8-3
	Bronchial swab	8-3
	Pericardial swab	7-3
6	Pericardial swab	7-3
	Joint	8-3
7	Nasal swab	10-4
	Bronchial swab	8-4
8	Lung	7-4
	Pleura	8-3
9	Joint	8-4
	Joint	9-4
10	Nasal swab	10-4
	Bronchial swab	8-4
11	Nasal swab	8-4
	Pleura	10-4
12	Pericardial swab	9-4
	Pleural Swab	7-4

*Gray area represents the animals that showed the same MLVA type in different sample types

CHAPTER III - Association between the number of tandem repeats in two important Mycoplasma hyopneumoniae adhesins

Dos Santos, L.F., et al., Association between the number of tandem repeats in two important Mycoplasma hyopneumoniae adhesins.

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Communication

[Comunicação]

Association of number of tandem repeats in two important adhesins in Mycoplasma hyopneumoniae

[Associação entre os números de repetições em tandem em duas importantes adesinas de Mycoplasma hyopneumoniae]

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Mycoplasma hyopneumoniae (M. hyopneumoniae) is the cause of enzootic pneumonia in pigs, one important disease that generates losses in swine production worldwide (Thacker and Minion, 2012). The success of M. hyopneumoniae colonization in the respiratory epithelium depends on the ability of this pathogen to attach to cilia (Mebus and Underdahl, 1977). Reduction in the adhesion capacity due to genetic variation could lead to decreased infectivity, as the loss of some of the adhesion proteins may end up in a reduction of the adherence ability of the mycoplasma (Razin, 1999).

In M. hyopneumoniae the adherence process is mainly mediated by receptor-ligand interactions and adhesins like P97 and P146 (Ferreira et al., 2007). Repeat region 1 (RR1), a locus containing tandem repeats (TR) in the gene mhp183 (P97) has been suggested as to be immunogenic and essential for ciliary binding (Wilton et al., 1998; Minion et al., 2000). The TR in RR1 can vary between strains and this variability can influence the ciliary binding (Wilton et al., 1998). At least eight repeats in RR1 have been suggested as needed for cilium binding, and three repeats are required for antibody recognition (Minion et al., 2000). In the gene mhp 684 (P146), the locus of the polyserine RR3 is of unknown function, although it is located in a P85 fragment that is associated with binding to porcine respiratory cilia and plasminogen (Bogema et al., 2012). Multiple-locus variable number tandem repeat analysis (MLVA) of RR1-P97 and RR3-P146 has recently demonstrated to be a useful tool for typing M. hyopneumoniae variants (Dos Santos et al., 2015). However, the usefulness of this approach to investigate the association between the two loci at the regional level has not been assessed. Moreover, little is known about the possible association between TR in RR1-P97 and in RR3-P146. Therefore, the aim of this study was to describe the spatial distribution and genetic heterogeneity of M. hyopneumoniae based on the TR in both loci in Brazil, and to investigate the association between RR1-P97 and RR3-P146.

One hundred fifty two clinical samples, submitted to the Microvet Laboratory (Viçosa, MG, Brazil) during Jan 2013 – March 2014 for diagnosis of M. hyopneumoniae were included in the analysis. Samples originated from 42 different production systems (n = 63 farms) located in eight states in Brazil, which included the main pig production areas (Figure 1). Samples that tested positive for M. hyopneumoniae by PCR were submitted to the Mycoplasma Research Laboratory at the University of Minnesota for MLVA typing, as previously described (Dos Santos et al., 2015). The number of repeats in each locus were recorded and combined to yield individual MLVA types. Spatial distribution of MLVA types was explored using ArcGIS 10.1 SP1 (ESRI, Redlands, CA, USA) and evidence of spatial clustering of profiles with given numbers of TR in RR1-P97 locus was assessed using the Bernouilli model of the spatial scan

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statistic (Kulldorff and Nagarwalla, 1995), implemented using the SaTScan software version 9.1.1. The relationship between the TR in the two loci was explored using non-parametric methods (Spearman correlation and Mann-Whitney tests) using the SPSS software V.22 (IBM Inc., Chicago, IL, USA). The Simpson's index of diversity (D) for MLVA types was calculated using the Hunter-Gaston method (Hunter and Gaston, 1988).

Figure 1. Distribution of MLVA types observed in 63 farms in Brazil. Repeat region 1 (RR1) P97 was categorized as high (≥ 8 tandem repeats, black stars) and low (< 8 tandem repeats, white circles). Representation of the MLVA types on the map is based on the actual herd location. The gray circle represents a significant (P=0.05) cluster of MLVA types with a low number of tandem repeats in RR1 P97 (observed to the expected ratio of 4.22). States identified with the two letter code are the most important areas of swine production in Brazil: Goiás (GO), Minas Gerais (MG), Mato Grosso (MT), Mato Grosso do Sul (MS), Paraná (PR), Rio Grande do Sul (RS), São Paulo (SP) and Santa Catarina (SC).

A total of 94 samples tested positive for M. hyopneumoniae and were further typed, yielding 39 MLVA unique types. One pattern (12-14) accounted for 32.9% of the samples. For the purpose of the spatial analysis, only unique MLVA types per site (farm) were considered (n = 76). MLVA types with high (≥8) TR in RR1-P97 were more abundant (61/76) and more

widely distributed across the 84 sampled population, while types with low (<8) TR were found in 19.7% of the samples and were located in two geographical areas, one of which included a significant (P=0.05) cluster of samples, with an observed to expected ratio of 4.22 (Figure 1). Most farms (49/63, 77.7%) submitted samples that yielded only high TR in RR1-P97, while 13 farms (20.6%) submitted samples in which only low TR RR1-P97 were detected. A combination of both high and low TR in RR1-P97 was identified in one farm only (Table 1).

A significant negative correlation (Spearman rho=-0.26; P=0.022) between TR in RR1-P97 and in RR3-P146 was observed. Samples with low TR in RR1-P97 showed a median of 26 TR in RR3-P146, while samples with high TR in RR1-P97 had a median of 15 TR in RR3-P146 (Mann-Whitney test, P=0.025).

A high heterogeneity of M. hyopneumoniae was observed in the sampled population, with 39 MLVA types from 76 farms (D=0.92). An identical MLVA type in a majority of the farms (33.3%, 21/63) could indicate a common source of infection. However, no information about the potential epidemiological connections between affected herds was available, thus the source of infection and mode of spread of the M. hyopneumoniae variants among herds are unknown. While MLVA types with high TR in RR1-P97 were widely distributed, farms in which only types with low TR in RR1-P97 were found were less abundant and clustered (Figure 1), which could suggest horizontal spread or exposure to a common local source. Variants with low TR in RR1-P97 may have an impaired adhesion capacity and therefore a decreased infectivity. Still, here low TR in RR1-P97 MLVA types were the only types found in 20.6% of the farms. Therefore, our results suggest that M. hyopneumoniae variants from Brazil with low TR in RR1-P97 could still be infectious, given that they were the only M. hyopneumoniae variants detected in certain clinically affected systems. The negative association of TR in RR1-P97 with in RR3-P146 may suggest a possible compensatory mechanism that would allow the bacterium to keep its full adhesion capacity even after the reduction of TR in RR1-P97. However, further studies should be performed to confirm this hypothesis. The results obtained in this investigation will contribute to understand the epidemiology and impact of M. hyopneumoniae in the main production areas in Brazil, one of the largest pig producing countries in the world.

Keywords: Adhesion, VNTR, MLVA, swine, enzootic pneumonia

RESUMO

Diversidade genética de Mycoplasma hyopneumoniae tem sido relatada em análise múltipla de repetições em tandem em número variável (MLVA). O objetivo deste estudo foi descrever a distribuição espacial e a heterogeneidade genética de tipos de M. hyopneumoniae no Brasil, bem como investigar a correlação entre regiões de repetição 1 (RR1) e 3 (RR3) de duas adesinas importantes (P97 e P146). Foram identificados 39 tipos de MLVA baseados no número de repetições em tandem em P97 RR1 e RR3 P146. A correlação negativa significativa (Spearman's rho = -0,26; P = 0,022) entre P97 RR1 e RR3 P146 foi observada, o que sugere um possível mecanismo compensatório que permitiria a bactéria manter a sua capacidade de adesão. Os resultados contribuem para compreender a epidemiologia das M. hyopneumoniae no quarto maior país produtor de suínos do mundo.

Palavras-chave: adesina, VNTR, MLVA, suíno, pneumonia enzoótica

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Table 1. Mycoplasma hyopneumoniae MLVA types identified in Brazil during Jan 2013 – March 2014.

Sample ID	No. of tandem repeats		State *
	RR1-P97	RR3-P146	
1	7	15	PR
2	7	22	MG
3	7	23	SC
4	7	24	SC
5	7	24	MG
6	7	24	MG
7	7	25	MG
8	7	26	MT
9	7	27	MG
10	2	29	RS
11	7	32	MG
12	7	33	SC
23	6	33	GO
14	7	34	SC
15	7	35	SC
16	12	13	MT
17	10	14	PR
18	10	14	MG
19	10	14	MG
20	12	14	MG
21	12	14	MG
22	12	14	SC
23	12	14	MS
24	12	14	SC
25	12	14	MG
26	12	14	MS
27	12	14	PR
28	12	14	MG
29	12	14	SP
30	12	14	SP
31	12	14	MG
32	12	14	MG
33	12	14	MG

Sample ID	No. of tandem repeats		State
	RR1-P97	RR3-P146	
40	12	14	GO
41	14	14	SC
42	8	15	SC
43	9	15	PR
44	12	15	MG
45	12	15	PR
46	12	15	MG
47	12	15	PR
48	10	16	MG
49	10	16	SC
50	10	17	RS
51	10	18	MG
52	10	18	MG
53	11	21	RS
54	11	21	RS
55	10	24	SC
56	12	31	PR
57	8	32	SP
58	11	32	SC
59	12	32	MG
60	12	32	SC
61	12	33	SC
62	12	33	SC
63	13	33	SC
64	13	33	PR
65	8	34	PR
66	12	34	RS
67	12	34	SC
68	11	35	GO
69	12	35	PR
70	12	35	PR
71	9	38	RS
72	12	38	SC

34	12	14	RS	**73**	12	38	RS
35	12	14	RS	**74**	12	38	SC
36	12	14	MG	**75**	8	41	MT
37	12	14	MT	**76**	14	48	MG
38	12	14	SC				
39	12	14	RS				

* States in Brazil: Goiás (GO), Minas Gerais (MG), Mato Grosso (MT), Mato Grosso do Sul (MS), Paraná (PR), Rio Grande do Sul (RS), São Paulo (SP) and Santa Catarina (SC).

General conclusions

Surveillance and control of emerging and re-emerging diseases with important economic losses is necessary to improve the profits of the producer and to the swine industry. Both, M. hyopneumoniae and M. hyorhinis are important pathogens that affect swine herds worldwide and have economic impacts in the production. The clinical course and the losses associated with both pathogens infections in a herd are dependent on many factors including herd management practices, environmental factors, secondary infections and the virulence of the strain. A better understanding in the diversity of mycoplasmas in the herd could elucidate questions in the epidemiology that are unclear. Therefore, the objective of this thesis was to investigate if a nonrandom distribution of genotypes of M. hyopneumoniae and M. hyorhinis strain vary according to distinct geographical regions exploring new typing approaches to be used to better understand the diversity and epidemiology of M. hyopneumoniae and M. hyorhinis.

Overall, this work demonstrated the practical applications of the MLVA approach to investigate the genetic diversity of both pathogens in the field. In the first study, the MLVA typing developed method is an improved assay for differentiation of M. hyopneumoniae variants in clinical specimens. This assay revealed a high discriminatory index, suggestive of high diversity of M. hyopneumoniae in the investigated herds, indicating that multiple M. hyopneumoniae variants are circulating in swine herds in the USA, Brazil, Mexico and Spain and no common MLVA type was observed in samples from all the regions included in this investigation. The MLVA assay proved to be an efficient tool for typing M. hyopneumoniae which can be standardized in diagnostic laboratories as it offers high stability and reproducibility. However, the fact that the equipment to perform the capillary electrophoresis and the hardware to analyze the results are costly, should be taken into account submit the samples to

facilities that own this apparatus. With the advance of the Whole Genome Sequencing (WGS), the comparison between the MLVA assay and WGS should be performed if a better characterization of the strain is necessary.

In the second study, the issue addressed was the heterogeneity of M. hyorhinis using a molecular tool based on the VNTRs present in the genome of this bacterium. An MLVA assay was developed, which was reproducible and easy to interpret. A limited number of M. hyorhinis MLVA types were identified in the set of isolates and clinical specimens used in this study. The heterogeneity of M. hyorhinis, observed in this investigation is in agreement with the results reported by Tocqueville et al. (2014). The study highlights that different MLVA types were identified in the same animal in different sample types. It would be interesting to evaluate this MLVA method in outbreak investigations to confirm the level of discrimination of this typing method.

And finally the third study, highlight the high heterogeneity of M. hyopneumoniae observed in Brazil, with 39 MLVA types from 76 farms An identical MLVA type in a majority of the farms (33.3%) could indicate a common source of infection. However, no information about the potential epidemiological connections between affected herds was available, thus the source of infection and mode of spread of the M. hyopneumoniae variants among herds are unknown. The variants with low tandem repeat in RR1 P97 may have an impaired adhesion capacity and therefore a decreased infectivity but can still be infectious in the field, given that they were the only M. hyopneumoniae variants detected in certain clinically affected systems. The negative association of TR in RR1-P97 with in RR3-P146 may suggest a possible compensatory mechanism that would allow the bacterium to keep its full adhesion capacity even

after the reduction of TR in RR1-P97. However, further studies should be performed to confirm this hypothesis.

The identification of the genetic heterogeneity of M. hyopneumoniae and M. hyorhinis will assist local epidemiological or outbreak investigations, design future control strategies as well as serve as a potential tool to study the evolutionary biology of this species.

The studies highlight the lack of country-wide epidemiological studies investigated. These studies can be used as a genotype comparison universally if a basic comparison is needed. For deeper analysis the WGS is more appropriate, however no studies about M. hyopneumoniae and M. hyorhinis WGS comparison has been done yet. This would make the process more laborious.

In conclusion, differential colonization between Mycoplasmas with different MLVA types and selection within Mycoplasmas with specific MLVA types should account for nonrandom genotype distribution among M. hyopneumoniae and M. hyorhinis variants observed at the spatial scale of this study.

Perspectives

The MLVA assays developed can be used to perform epidemiological investigations, which will investigate the genetic variability of both Mycoplasmas within farms and among farms. Also the MLVA assay could be used to investigate an outbreak, and identify of the potential origin of a specific isolate. Would be interesting investigate correlation between the clinical presentation of the disease and the MLVA types of both Mycoplasma to verify if some MLVA type can cause a more severe disease than others type. That information will be used to acquire new knowledge with regard to M. hyorhinis and M. hyopneumoniae infections.

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