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# Face to face with *Actinobacillus pleuropneumoniae*: Landscape of the distribution of clinical isolates in Southeastern Brazil

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**Pleuropneumonia caused by *Actinobacillus pleuropneumoniae* is one of the most devastating diseases in the scenario of swine global production, in which Brazil occupies a prominent place. *A. pleuropneumoniae* have been shown to be difficult to type by the existing methods given its existence in 15 different serotypes. The goals of this study were to establish an identification experimental strategy of *A. pleuropneumoniae* serotypes by multiplex PCR with *apx* genes and PCR-REA-*apxIVA* gene and evaluate the potential of ERIC, BOX and $(GTG)_5$-PCR as tools for molecular typing of *A. pleuropneumoniae* isolates obtained from swine with clinical signs of pleuropneumonia in southeastern Brazil. Multiplex PCR and PCR-REA allowed the characterization of 95% of the isolates, with prevalence of band patterns equal to serotype reference strains 2, 7 and 8. Although, serotype 8 was not the most abundant, it was certainly the most widespread. The absence of predominant genotypes groups as observed by the *rep* fingerprinting and the emergence of different isolates throughout the years suggest molecular events that led to the great genetic variability observed. Our results display an effective strategy in the study of different serotypes of *A. pleuropneumoniae* and reveal an updated vision of the distribution and genomic fingerprinting of clinical isolates in southeastern Brazil.**

**Key words:** *Actinobacillus pleuropneumoniae*, PCR-REA, ERIC-PCR, BOX-PCR, $(GTG)_5$-PCR.

## INTRODUCTION

In the past 30 years, Brazilian pork production raised from 1.096 to 3.384 million tonnes, with an annual expansion of 3.7% in this period and still experiences constant growth in international markets (Pereira et al., 2012). Despite of the economic advances, swine pleuropneumonia causes significant economic losses in all pig-producing countries. The Gram-negative bacterium *Actinobacillus pleuropneumoniae* is the causative agent of the disease and is divided into two biotypes based upon the growth requirement of nicotinamide adenine dinucleotide (NAD) and 15 different serotypes defined based on antigenic properties of capsule polysaccharides (Blackall et al., 2002). Although, the bacterial virulence is multi-factorial, the severity of the disease caused by *A. pleuropneumoniae* is strongly related to the production and secretion of a variety of exotoxins of the repeat in toxin (RTX) family (*apx* genes), which present a range of degrees of hemolytic and cytotoxic activities and are produced in different combinations by the 15 serotypes (Frey, 2011). Several molecular strategies based on poly-

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merase chain reaction (PCR) using the *apx* genes have been developed to detect, genotype and characterize *A. pleuropneumoniae*, but they fail to differentiate all the 15 serotypes (Gram et al., 2000; Guo-Sheng et al., 2006; Rayamajhi et al., 2005). Nonetheless, the ease and low cost of these techniques have made them key factors in diagnosis and the epidemiological study (Dubreuil et al., 2000). Moreover, the analysis of the presence and distribution of repetitive elements (*rep*-PCR or *rep* fingerprinting) in the genomes of microorganisms has proven to be an advantageous tool in the study of variability in microbial isolates, microbial ecology, environmental microbiology, molecular diagnosis, medical microbiology and epidemiological analyses (Ishii and Sadowsky, 2009). Three of these analytical methods that target distinct repetitive sequences are ERIC-PCR, BOX-PCR and $(GTG)_5$-PCR, which have been demonstrated to enable the differentiation of organisms at the level of subspecies and even strain (Healy et al., 2004; Wise et al., 2007).

There are not many consistent studies on the current scenery of swine pleuropneumonia in Brazilian farms and according to our knowledge there are no studies concerning the disease in the southeastern of the country, which plays an important role in Brazilian swine industry. The objectives of this study were to establish and execute an efficient strategy to genotype *A. pleuropneumoniae*, accessing their presumable serotypes and genetic variability, employing clinical isolates obtained in southeastern Brazil.

## MATERIALS AND METHODS

### Microorganisms and growth conditions

In this study, 96 *A. pleuropneumoniae* strains (16 serotype reference strains and 80 clinical isolates) were used. Six clinical isolates of *Haemophilus parasuis* (3) and *Pasteurella multocida* (3), both also from *Pasteurellaceae* family were adopted as experimental control (Table 1). The clinical isolates of *A. pleuropneumoniae* were obtained from the lungs, lung abscesses or tonsils swabs of swine with clinical manifestation of pleuropneumonia between the years of 2003 and 2010 (with exception of isolate MV010, obtained in 1996) from nine different farms, designated as *A* to *I*. The three major productive areas of the region are included in the study; therefore, this sample can be an approximate representation of the population of swine *A. pleuropneumoniae* related to clinical outbreaks in southeastern Brazil. All the clinical isolates (*A. pleuropneumoniae*, *H. parasuis* and *P. multocida*) were kindly provided by the company MICROVET. The same company kindly provided the genomic DNA of the serotype reference strains.

Subclinical infections were not evaluated in this study. All isolates were grown at 37°C for 24 h at 5% $CO_2$ in Brain Heart Infusion (Oxoid, Hampshire, United Kingdom) supplemented with NAD (10 $\mu g.ml^{-1}$ - Sigma-Aldrich, Poole, United Kingdom). All the experiments were conducted from bacterial stocks kept at -80°C.

### DNA preparation

Genomic DNA was isolated with the Wizard® Genomic DNA Purification Kit (Promega, Madison, USA) following the manufacturer's instructions. The DNA samples obtained were analyzed by electrophoresis in agarose gel (0.8%) and by spectrophotometry. All the molecular biology procedures described as follows were standardized and repeated at least three times independently to ensure reproducibility of methods.

### Multiplex PCR based on *apx* genes

The multiplex PCR reaction of the *apx* genes was conducted according to Rayamajhi et al. (2005), performed in a thermal cycler C1000™ (BioRad, Richmond, USA) using the oligonucleotide pairs APXIAF/APXIAR, APXIBF/APXIBR, APXIIF/APXIIR, APXIIIF/APXIIIR and APX4DWN-L/APXIV-IR (Table 2).

### PCR-restriction enzyme analysis (PCR-REA)

The *apxIVA* gene of clinical isolates with inconclusive band patterns (equal to more than one serotype reference) by multiplex PCR based on *apx* genes were amplified according to the methods suggested by Jaglic et al. (2004), using the primer pair APXIVAF/APXIVAR (Table 2). The amplified product was analyzed and quantified by electrophoresis in 1.0% agarose gel, and 1 µg of the PCR product was used for endonuclease digestion with one of the three enzymes *Alu*I, *Hha*I and *Hpa*II (BioLabs, Massachusetts, USA). The reaction was performed according to the manufacturer's instructions. The size of the DNA fragments and the profile of the fragments generated for the isolates were compared to the fragment profiles of the serotype reference strains.

### *Rep* fingerprinting: ERIC-PCR, BOX-PCR and $(GTG)_5$-PCR

The three *rep-PCR* reactions were performed in the C1000™ Thermal cycler (BioRad, Richmond, USA) using 1 U of JumpStart™ *Taq* DNA Polymerase (Sigma-Aldrich, Poole, United Kingdom). The primers used are listed in Table 2. The reaction and cycle parameters of the *rep*-PCR reactions were conducted according to Mohapatra and Mazumder, (2008). The amplified fragments were separated by electrophoresis in a 2.0% agarose gel. The fingerprinting patterns obtained were analyzed using the software BioNumerics 5.0 (Applied Maths, Sint-Martens-Latem, Belgium) and used in the construction of dendrograms by the UPGMA method. The cophenetic correlation coefficient (Farris, 1969) was calculated to test the goodness of fit between the similarity showed in the dendrograms and the cophenetic matrices.

## RESULTS

### Identification of the serotypes of *Actinobacillus pleuropneumoniae* clinical isolates: *apx* genes multiplex PCR and PCR-REA

The presumable serotypes of eighty clinical isolates of *A. pleuropneumoniae* were determined by means of multiplex PCR based on *apx* genes complemented with PCR-REA with the gene *apxIVA*. The fragment profiles obtained for the clinical isolates were compared to the serotype references strains and one of the isolates showed a band pattern equal to the serotype reference 1, four equal to serotype reference 5 and thirty-five equal to serotype reference 7. Four isolates (5.0%) did not display

**Table 1.** Bacterial strains and clinical isolates used in this study.

| ***Actinobacillus pleuropneumoniae*** | | |
|---|---|---|
| **Origin** | | **Isolates (year of isolation)** |
| **São Paulo** | | |
| | Farm A | MV573, MV6584 (2006) |
| **Minas Gerais** | | |
| Region 1 | | |
| | Farm F | MV344, MV345, MV5237 (2005); MV512, MV555 (2007) |
| Region 2 | | |
| | Farm C | MV433 (2006) |
| | Farm G | MV5264 (2008) |
| | Farm I | MV710 (2008) |
| Region 3 | | |
| | Farm B | MV496, MV505 (2007); MV653 (2008); MV732 (2009) |
| | Farm D | MV513 (2007) |
| | Farm E | MV215, MV216, MV222, MV223, MV224, MV225 (2002); MV235, MV3174, MV3440 (2003); MV263, MV276, MV277, MV278, MV279, MV280, MV284, MV294, MV297, MV3571 (2004); MV313 (2005); MV368, MV370, MV384, MV407, MV5651 (2006) |
| | Farm H | MV010 (1996); MV234, MV236 (2003); MV374, MV375, MV376, MV377, MV378, MV396, MV403, MV404, MV405, MV406, MV432, MV440, MV442, MV443 (2006); MV452, MV470, MV471, MV473, MV485, MV486, MV487, MV492, MV497, MV540 (2007); MV718, MV622, MV623, MV688, MV696 (2008); MV742, MV804, MV807, MV808, MV828, MV831 (2009), MV836 (2010) |
| **Reference strains (serotype)** | | |
| | | Shope4074 (01), S1536 (02), S1421 (03), M62 (04), K17 (5a), L20 (5b), FemΦ (06), WF83 (07), 405 (08), CVJ13261 (09), D13039 (10), 56153 (11), 8329 (12), N-273 (13), 3606 (14), HS143 (15) |
| **Other species** | | |
| *Haemophilus parasuis* | | 9190, 9400, 10005 |
| *Pasteurella multocida* | | 1769, 5869, 5875 |

**Table 2.** Oligonucleotides used in this study.

| Name | Sequence (5'-3') | Genbank access | Position | Amplicon (pb) | Source |
|---|---|---|---|---|---|
| APXIAF | atcgaagtacatcgctcgga | X52899 | 275-295 | 723 | Rayamajhi et al. (2005) |
| APXIAR | cgctaatgctacgaccgaac | X52899 | 968-998 | | |
| APXIBF | ttatcgcactaccggcactt | X68595 | 4102-4121 | 811 | |
| APXIBR | tgcagtcaccgattccacta | X68595 | 4893-4913 | | |
| APXIIF | gaagtatggcgagaagaacg | AY736188 | 973-993 | 965 | |
| APXIIR | cgtaacaccagcaacgatta | AY736188 | 1918-1938 | | |
| APXIIIF | gcaatcagtccattggcgtt | X80055 | 9558-9578 | 396 | |
| APXIIIR | gacgagcatcatagccattc | X80055 | 9934-9954 | | |

**Table 2.** Contd

| | | | | | |
|---|---|---|---|---|---|
| APX4DWN-L | gcgaaacaattcgaaggg | AF021919 | 6459-6442 | 2331 | Sthitmatee et al. (2003) |
| APXIV-IR | ggccatcgactcaaccat | AF021919 | 4111-4128 | | |
| APXIVAF | gcctccgacctgaataaacc | AF021919 | 2923-2942 | 3525 | Jaglic et al. (2004) |
| APXIVAR | caaccatcttctccacc | AF021919 | 6432-6448 | | |
| ERIC1R | atgtaagctcctggggattcac | - | - | variable | Versalovic et al.(1994) |
| ERIC2 | aagtaagtgactggggtgagcg | - | - | | |
| BOXA1R | ctacggcaaggcgacgctgacg | - | - | | |
| $GTG_5$ | gtggtggtggtggtg | - | - | | |

**Table 3.** Determination of the presumable serotypes of clinical isolates of *Actinobacillus pleuropneumoniae* used in this study.

| Isolate | Reference serotype band pattern | |
|---|---|---|
| | Multiplex PCR | PCR-REA |
| MV010 | 1 | 1 |
| MV496, MV653, MV732, MV505 | 5 | 5 |
| MV234, MV236, MV344, MV345, MV384, MV433, MV512, MV513, MV573, MV555, MV5237, MV5264, MV5651, MV6584 | 2, 8 or 15 | 8 |
| MV215, MV216, MV222, MV223, MV224, MV225, MV284, MV294, MV263, MV276, MV277, MV278, MV279, MV280, MV297, MV313, MV368, MV370, MV407, MV3174, MV3440, MV3571 | 2, 8 or 15 | 2 |
| MV374, MV375, MV376, MV377, MV378, MV396, MV403, MV404, MV405, MV406, MV432, MV440, MV442, MV443, MV470, MV471, MV473, MV485, MV486, MV487, MV492, MV540, MV622, MV623, MV688, MV696, MV710, MV742, MV804, MV807, MV808, MV828, MV831, MV836 | 7 | 7 |
| MV235, MV452, MV497, MV718 | NG | NG |

NG: Isolate non-genotypable by the molecular technique applied.

band patterns that were comparable to any of the references and were considered to be non-genotypable. The thirty six remaining isolates classified by multiplex PCR showed band patterns equal to either serotype reference strains 2, 8 or 15. Their discrimination was only possible by complementing the results employing PCR-REA. Either one of the three enzymes chosen could successfully distinguish between the three serotypes and among those thirty six isolates, 39% displayed band patterns equal to serotype reference 8 and 61% equal to serotype reference 2. Table 3 summarizes the results for both PCR techniques employed in the classification of *A. pleuropneumoniae* clinical isolates of this study.

Overall, the profile of isolates of our sample was presumably of 43.7% isolates of serotype 7; 27.5% of serotype 2; 17.5% of serotype 8; 5.0% of serotype 5; 1.3% of serotype 1 and the remaining 5.0% were classified as non-genotypable isolates. Some serotypes are farm-specific (such as serotype 2 in farm E) and serotype 8 appears to be the most widespread because isolates with its band pattern were found in almost every farm analyzed.

## Characterization of clinical isolates of *A. pleuropneumoniae* by *rep* fingerprinting

Three *rep* fingerprinting methods with distinct DNA targets were applied to investigate the genetic variability of *A. pleuropneumoniae* clinical isolates found in the

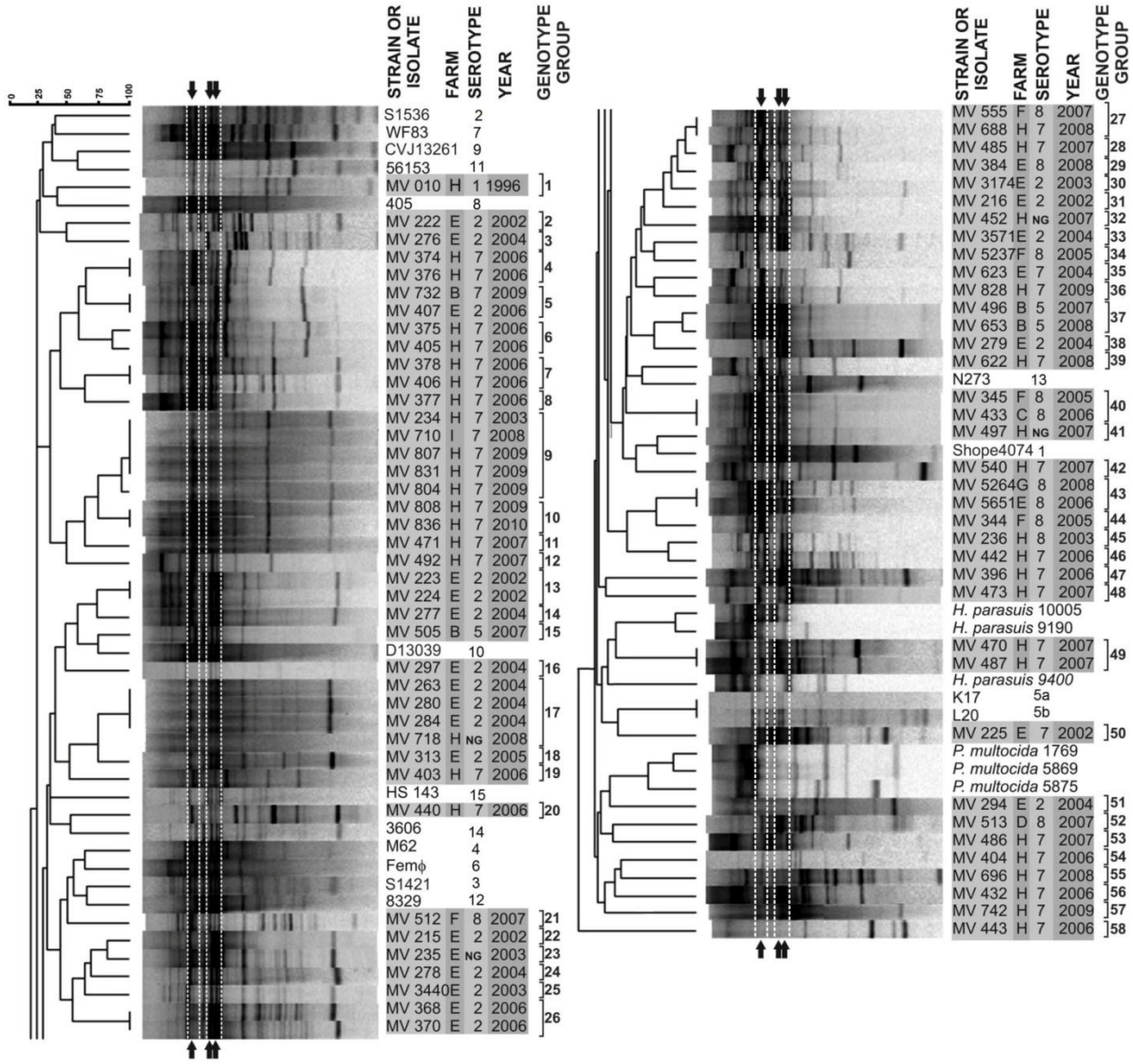


**Figure 1.** Cluster analysis of ERIC-PCR fingerprints of the clinical isolates *Actinobacillus pleuropneumoniae* in southeastern Brazil. The name of the isolate or strain is shown along with the serotype (or presumable serotype as defined by PCR techniques), farm, year of isolation and genotype group. Arrows show DNA fragments common to most of *A. pleuropneumoniae* isolates.

Southeast Brazil. By using the standardized conditions, all reactions were reproducible and the three dendrograms were supported statistically by a high cophenetic correlation value, all greater than 0.85. Still, to avoid misinterpretations of band patterns, a 90% cut-off was adopted to divide the isolates in specific genotype groups. Unexpectedly, there are not prevalent groups among our isolates, as great number of them was found for the three techniques: 58 for ERIC-PCR (Figure 1), 53 for BOX-PCR (Figure 2) and 60 for $(GTG)_5$-PCR (Figure 3). The genotype groups generally comprise microorganisms of same presumable serotype and origin, but many isolates with the same characteristics are displayed in distant groups. It shows that even though same serotype isolates are more similar, they have also suffered genetic modifications that separated them from each other within their population in the farms analyzed. This is reinforced by the aggregation of the isolates in the dendrograms independent from the serotype references as well as the strains of different species.

Although, it was not our main objective, the techniques also reveal the possibility of assessing the dynamics of the population for epidemiological studies, since it is clear the aggregation of some isolates according to their year

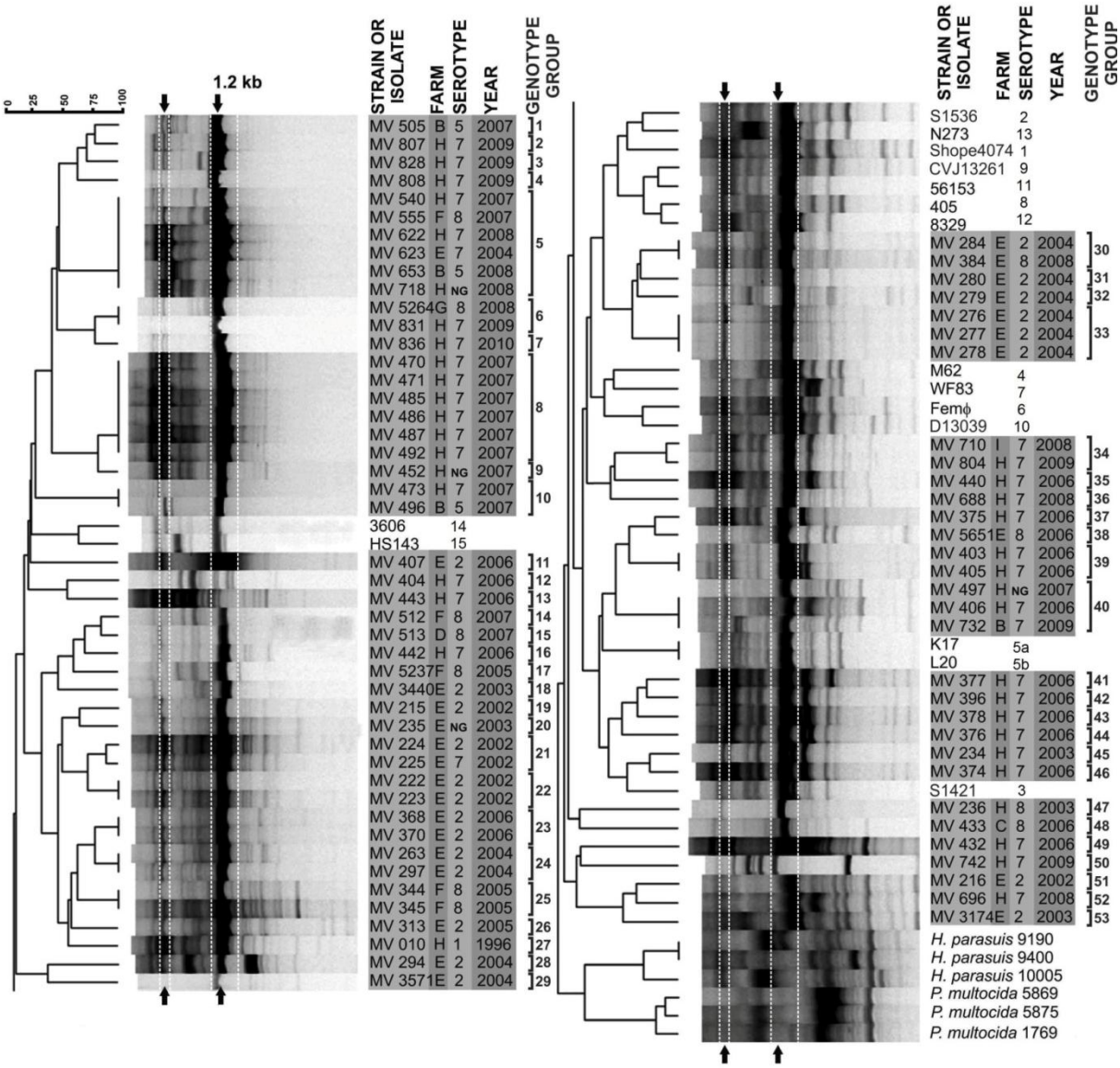


**Figure 2.** Cluster analysis of BOX-PCR fingerprints of clinical isolates *Actinobacillus pleuropneumoniae* in southeastern Brazil. The name of the isolate or strain is shown along with the serotype (or presumable serotype as defined by PCR techniques), farm, year of isolation and genotype group. Arrows show DNA fragments common to most of *A. pleuropneumoniae* isolates.

of isolation in a specific farm (as it can be seen for isolates of serotype 7 from farm H). Moreover, the use of three *rep* fingerprinting methods shows complementary results: isolates designated as identical in one technique (for example, MV223 and MV224 in ERIC-PCR) have some minor differences revealed in the others (Figures 1 to 3). The bands profiles obtained for BOX-PCR shown in Figure 2 emphasize the existence of a frequent and prominent DNA fragment of approximately 1200 bp, specific for *A. pleuropneumoniae*. Frequent fragments are observed in the three dendrograms, but the one in BOX-PCR has particularly been observed for 96.8% of the *A. pleuropneumoniae* isolates and references studied and none of the other *Pasteurellaceae* family members.

## DISCUSSION

The discrimination between the 15 serotypes of *A. pleuropneumoniae* by molecular methods has been the objective of several studies. They usually aim to complement or replace data from serological assays, since the latter often result in cross-reactions. Most of the molecular methods involve multiplex PCR reactions with

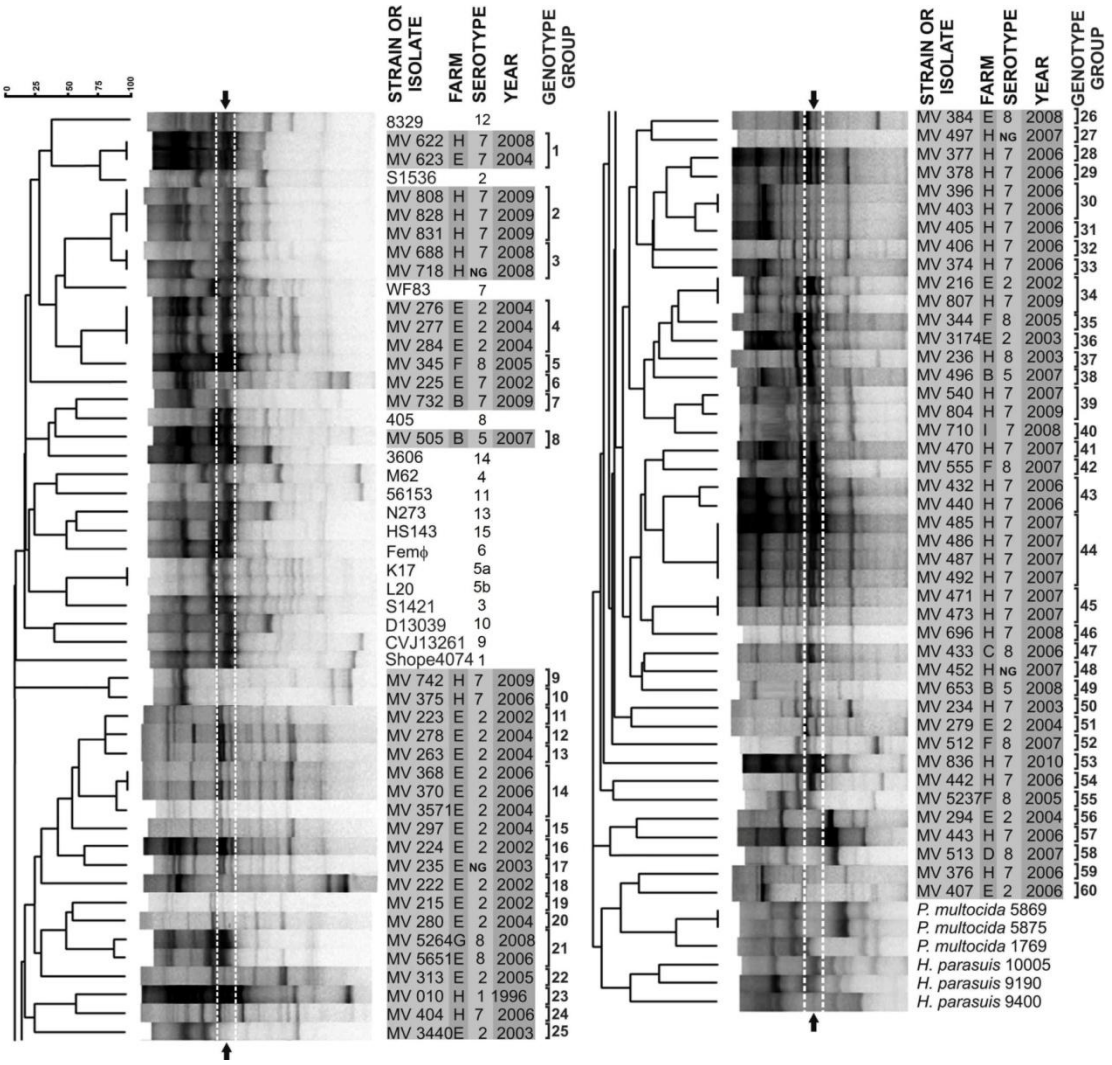


**Figure 3.** Cluster analysis of $(GTG)_5$-PCR fingerprints of clinical isolates *Actinobacillus pleuropneumoniae* in southeastern Brazil. The name of the isolate or strain is shown along with the serotype (or presumable serotype as defined by PCR techniques), farm, year of isolation and genotype group. Arrows show DNA fragments common to most of *A. pleuropneumoniae* isolates.

either the exotoxin-coding *apx* genes (Sthitmatee et al., 2003; Tonpitak, 2009) or the *cps* genes, involved in the biosynthesis of the capsular polysaccharides (Jessing et al., 2003; Schuchert et al., 2004). Although, many of these reactions are capable to differentiate specific serotypes, they fail to obtain conclusive results for all the 15 serotypes. Given a partially unknown sample like ours, we chose to use the method developed by Rayamajhi et al. (2005) because it is one of the reactions we agreed that could discriminate the greatest number of serotypes. It is, therefore, a fine approach to start the work in describing an uncharacterized sample. Based on the fact that a fraction of our isolates were indistinguishable between serotypes 2, 8 or 15, we decided to complement our data with PCR-REA with the *apxIVA* gene. This technique developed by Jaglic et al. (2004) and employed by Turni and Blackall (2007) has also been proven to be not efficient to differentiate all the 15 serotypes by itself. But in the present work, we showed that PCR-REA can be successfully used with any of the three enzymes *Alu*I, *Hha*I and *Hpa*II to separate clinical isolates of *A. pleuropneumoniae* of serotypes 2, 8 or 15, giving definitive results that were coherent with the ones obtained with multiplex PCR.

Overall, 95% of our isolates were effectively characterized by using the two molecular techniques we proposed. The remaining 5% gave band patterns that could not be compared to the serotype references and signal genetic variability among the isolates. It is still a great result, even more if we compare it to a previous work applied to other Brazilian clinical isolates. In the referred research, 399 isolates were serotyped by immu-

nodiffusion, but 171 (42.8%) of them could not be classified due to cross-reactivity or loss of capsule production by the cultures (Kuchiishi et al., 2007).

The swine industry in the southeast of Brazil is mainly composed by small and medium properties which generally work independently of each other, avoiding cross-contamination of herds. It is probably the reason why some serotype-related band patterns obtained by multiplex PCR and PCR-REA are characteristic of each farm, such as serotype 5 in farm B, serotype 7 in farm H and serotype 2 in farm E. Our results, however, point to the fact that historically, serotype 8 may have been widespread in the region analyzed, as it is found in almost every farm sampled. Although, it is apparently not the most prevalent serotype, it must surely be the most persistent throughout the years. It differs from other countries such as the United States and China where the most important are serotypes 1, 5b and 7 in the first and serotype 3 in the latter (Dubreuil et al., 2000; Xu et al., 2008).

Even though the independency of the farms avoided cross-contamination in the pig production chain analyzed, the *rep* fingerprinting reactions revealed that isolates from the same farm still present a great genetic variability among themselves. This is the first work to show the suitability of $(GTG)_5$-PCR and BOX-PCR in displaying the variations in the genomes of *A. pleuropneumoniae*. ERIC-PCR had already been employed as a typing method for *A. pleuropneumoniae* by Givisiez et al. (2006), but the study was limited to a small number of isolates, all of serotype 2. Reproducibility is a key factor in determining *rep* PCR dendrograms, and clustering algorisms help to increase the accuracy of the analyses (Ishii and Sadowsky, 2009). Here, the software 'bionumerics' was used to guarantee a more accurate result and reproducibility was evaluated by repeating reactions in independent runs. The three dendrograms were supported statistically by a high cophenetic correlation value, of more than 0.85. Furthermore, the 90% cut-off, which had already been adopted for *Haemophilus parasuis* (Macedo et al., 2011), was also applied to diminish the possibility of data misinterpretations.

The great amount of genotypes obtained, even among isolates of same serotype or farm suggests that this bacterium is subjected to intrinsic or environmental factors that may help it to increase its genetic variability. The high number of copies of the transposable element ISApl*1* in the genome of *A. pleuropneumoniae* may be one of these intrinsic factors (Liu et al., 2008) which may act not only as it transposes, as it also may increase the possibility of homologous recombination. *A. pleuropneumoniae* is also capable of natural transformation and its environment within the swine microbiota may also be an important variability factor (Bosse et al., 2004). The high intra-species variability highlights the importance of a constant surveillance of the pathogen population, since it is an important obstacle to the development of vaccines against several microbial diseases (Lemaire et al., 2012). That is possibly one of the reasons why autogenic vaccines have been employed more successfully in our region of study. In conclusion, our data propose and encourage a strategy for the determination of the serotypes of *A. pleuropneumoniae* based on multiplex PCR followed by PCR-REA and shows the applicability of ERIC-PCR, BOX-PCR and $(GTG)_5$-PCR in providing information concerning the genetic variability of clinical isolates.

Additionally, the presence of a prominent DNA fragment of approximately 1200 bp specific for *A. pleuropneumoniae* can represent a new molecular marker for the detection of the pathogen and its development is underway.

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