

Phylogenetic analysis of *Pneumocystis* from pig lungs obtained from slaughterhouses in southern and midwestern regions of Brazil

[Análise filogenética de *Pneumocystis* obtidos de pulmões de suínos provenientes de abatedouros da região Sul e Centro Oeste do Brasil]

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ABSTRACT

The *Pneumocystis* genus is comprised of pathogens dwelling in the lungs of terrestrial, aerial, and aquatic mammals. Occasionally they induce severe pneumonitis, particularly in hosts with severe impairment of the immune system and progressively may fill pulmonary alveolar cavities causing respiratory failure. Molecular genetic studies revealed that *Pneumocystis* gene sequences present a marked divergence with the host species concerned. In the present study, the genetic diversity of *Pneumocystis* obtained from lungs of swines was examined by analyzing *mitochondrial large subunit (mtLSU)* and *small subunit (mtSSU) rRNA* sequences. The samples were obtained from two slaughterhouses located in two Brazilian states. Phylogenetic analysis demonstrated that genetic groupings within *Pneumocystis* organisms were in accordance with those of the corresponding hosts and that two clusters were formed. In conclusion, these data show that there are genetically distinct porcine *Pneumocystis* genotypes with at least two separate clusters in Brazil.

Keywords: swine, *Pneumocystis*, phylogeny, *mitochondrial large subunit*, *small subunit*, *rRNA*

RESUMO

O gênero *Pneumocystis* compreende patógenos que residem em pulmões de animais terrestres, aéreos e aquáticos. Pode ocasionar uma grave pneumonia, particularmente em hospedeiros com o sistema imunológico seriamente comprometido, o que ocorre por meio de uma progressiva disseminação nas cavidades alveolares, causando insuficiência respiratória. Estudos genéticos, baseados em métodos moleculares, revelaram que as sequências dos genes de *Pneumocystis* apresentam marcante divergência de acordo com a espécie de hospedeiro. Neste estudo, a diversidade genética das amostras obtidas a partir de pulmões de suínos, provenientes de dois abatedouros localizados em dois estados brasileiros, foi examinada por análise das sequências dos nucleotídeos dos produtos de PCR dos genes *mtLSU* e *mtSSU* do *rRNA* do *Pneumocystis*. O resultado confirma a tendência registrada em pesquisas com amostras de outros animais e permite concluir que existem, pelo menos, dois grupos filogenéticos distintos de *Pneumocystis* de suínos no Brasil.

Palavras-chave: *Pneumocystis*, suínos, filogenia, *mitochondrial large subunit (mtLSU) rRNA*, *small subunit (mtSSU) rRNA*

Recebido em 11 de agosto de 2010

Aceito em 12 de agosto de 2011

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INTRODUCTION

Pneumocystis is a group of organisms assigned to the Fungal Kingdom (Edman *et al.*, 1988; Wakefield *et al.*, 1992; Calderon-Sandubete *et al.*, 2002). The genus comprises pathogens dwelling in the lungs of terrestrial, aerial, and aquatic mammals (Laakkonem e Sukura, 1997; Mazars *et al.*, 1997; Laakkonem, 1998; Guillot *et al.*, 1999; Durand-Joly *et al.*, 2000; Demanche *et al.*, 2001; Guillot *et al.*, 2001; Laakkonem *et al.*, 2001). Occasionally they induce severe pneumonitis, particularly in hosts with severe impairment of the immune system. In such hosts, *Pneumocystis* species develop progressively and may fill pulmonary alveolar cavities, a process that leads to respiratory failure (Dei-Cas, 2000).

Molecular genetic studies revealed that *Pneumocystis* gene sequences (*mtLSU rRNA*, *mtSSU rRNA*, *ITS*, *DHPS*, and *MSG*) present a marked divergence with the host species concerned. Numerous *Pneumocystis* gene fragments from humans and other primates, rodents, rabbits and insectivores have been compared. Consistently it was found that specific sequences could be attributed to pathogens from different hosts (Banerji *et al.*, 1995; Mazars *et al.*, 1995; Wakefield, 1998; Laakkonen, 1998; Denis *et al.*, 2000; Durand-Joly *et al.*, 2000; Guillot *et al.*, 2001).

A more recent study in primates showed that a large sequence divergence in mitochondrial ribosomal DNA subunits (*mtLSU rRNA*) among *Pneumocystis* species was correlated with the phylogeny of their hosts. This observation, which could be extended to other mammals (Demanche *et al.*, 2001; Guillot *et al.*, 2001), suggests that cophylogeny can explain the current distribution of pathogens in their hosts (Hugot *et al.*, 2003).

The high divergence among *Pneumocystis* species probably results from a prolonged process of coevolution with each mammal host and is mostly associated with cospeciation (Hugot *et al.*, 2003).

The present paper focuses on a *Pneumocystis* species identified through aligned DNA sequences of two genes *mtLSU-rRNA* and a small subunit of mitochondrial ribosomal RNA (*mtSSU-rRNA*) from lung samples of swine slaughtered in the regions of Porto Alegre, RS, and Cuiabá, MT, Brazil.

MATERIALS AND METHODS

The lung tissue samples were obtained from two slaughterhouses in separate regions of Brazil. Those from the Southern state of Rio Grande do Sul were designated RS, and those from the mid-western state of Mato Grosso were designated MT. Pig lungs were examined; those with lesions were identified and the areas taken by lesions were measured and recorded as percent of the total lung. Among 281 lungs collected in RS, 142 (50.5%) showed macroscopic signs of inflammation and consolidation of different grades of pneumonia. Likewise, among 283 lungs obtained in MT, 141 (49.8%) lungs showed similar lesion patterns. The overall rate of *Pneumocystis* positive samples detected was 36.9% (39.9% in RS and 33.9% in MT). Collected lung tissue samples were frozen after macroscopic examination and stored at - 20°C for DNA extraction (Cavallini Sanches *et al.*, 2006; 2007).

For *Pneumocystis* DNA extraction and PCR a small part of the lung tissue (from 250mg) was then finely minced, homogenized through crushing and analyzed with the Kit Qiagen (Dneasy™ tissue Kit, Qiagen, Courtaboeuf, France). DNA was extracted from 34 randomly selected samples of frozen lung tissues of 208 swine with *Pneumocystis* previously detected by Grocott and Immunohistochemical analysis. The presence of *Pneumocystis* DNA was determined by nested PCR at the *mtLSU rRNA* gene and a small subunit of mitochondrial ribosomal RNA (*mtSSU-rRNA*). For gene amplification (*mtLSU-rRNA*), the primer sets pAZ102H-pAZ102E and pAZ102X/R1-pA102ZY/R1 were used. Thermocycling conditions for the first PCR round were as follows: each cycle consisted of denaturation for 1.30 min at 94°C, annealing for 1.30min at 55°C, and extension for 2min at 72°C; 40 cycles were included in the protocol. The second round of PCR was performed with the primer pair pAZ102Y/R1 and pAZ102X/R1. Ten amplification cycles were carried out at 94°C for 1.30min, 55°C for 1.30min, and 72°C for 2min, followed by 30 cycles at 94°C for 1.30min, 63°C for 1.30min, and 72°C for 2min. Expected product sizes were approximately 350bp and 230bp for the first and second rounds of PCR, respectively (Cavallini Sanches *et al.*, 2007).

For the amplification of the *mtSSU-rRNA* gene, the primer sets pAZ102 10F-RI -pAZ102 10R-RI and pAZ102 13-RI -pAZ10214-RI were used in primary PCR. Thermocycling conditions for the first PCR round were as follows: each cycle

consisted of denaturation for 2min at 94°C, annealing for 1.30min at 55°C, and extension for 2min at 72°C; 40 cycles were included in the protocol. The second round of PCR was performed with the primer pair pAZ102 13-RI – pAZ10214-RI. Ten amplification cycles were carried out at 94°C for 1.30min, 52°C for 1.30 min, and 72°C for 2min, followed by 30 cycles at 94°C for 1.30min, 63°C for 1.30min, and 72°C for 2min. Expected product sizes were approximately 400bp and 300bp for the first and second rounds of PCR, respectively.

Amplification products were directly sequenced from both ends using the sets of internal primers on an automated DNA sequencer (GenomeExpress, Montreuil, France) at École Nationale Vétérinaire d'Alfort, Maison-Alfort, France.

For each species, *Pneumocystis mtSSU* and *mtLSU rRNA* sequences were combined. The sequences were initially aligned with the computer program CLUSTAL X (version 1.63b, December 1997) and then through visual optimization with a computer program using Se-Al version 0.1 (available on <http://envolve.zoo.ox.ac.uk/>). Regions that contained alignment ambiguities were systematically excluded from the phylogenetic analysis. Both the branch-and-bound and heuristic search options in the Phylogenetic Analysis Using Parsimony Program (PAUP 4.0) were used for comparison of sequence alignments and generation of parsimonious trees. The strength of the internal branches from the resulting trees was statistically tested by bootstrap analysis using 500 bootstrap replications.

RESULTS AND DISCUSSION

The sequences of genes for ribosomal RNA are particularly valid due to the codifying regions that are found in all organisms. The small subunit of ribosomal RNA (*16S-like rRNAs*) has gained importance due to the acceptance by inference of reliable structures for phylogenetic analyses. This region (*16S rRNA*) is formed by mosaics of genetic elements which show specific

rates of evolutionary mutation (Edman *et al.*, 1994).

In this report, the genetic diversity of *Pneumocystis* from swine is examined by analyzing mitochondrial large subunit (*mtLSU*) and small subunit (*mtSSU*) *rRNA* sequences.

The phylogenetic analysis demonstrated that genetic groupings within *Pneumocystis* organisms were in accordance with those of the corresponding hosts. After results from all analysis performed (neighbor joining, heuristic search and branch and bound), two clusters were observed. The first cluster was composed of *Pneumocystis* organisms from PN25P and PN64P swine in Porto Alegre and Cuiabá regions. The second cluster was composed of *Pneumocystis* organisms of 32 swine (PN7C, PP6C, PP82P, PP50C, PP107C, PN4C, PN67P, PP65, PN105P, PN36, PN73P, PN80P, PP77P, PPCBA46, PPPOA38, PN86P, PN5P, PPCBA6, PP69C, PPPOA65, PPPOA61, PP38, PN117, PNPOA56, PNPOA9, PN91P, PPBA69, PN76P, PP5C, PPCBA50, PN59, PP102), from the same distinct geographical regions.

In the first cluster, swine without macroscopic lesions in lungs and the second cluster were either with or without pneumonia.

The use of mitochondrial genes as phylogenetic markers shows that in the *mtSSU rRNA* gene supported by a bootstrap value (Fig. 1) and *mtLSU rRNA* was supported by a variable (low) bootstrap value (Fig. 2).

These data constitute the first report on *Pneumocystis* colonizing the lungs of pigs in Brazil, also showing that porcine *Pneumocystis* sp. are genetically distinct, suggesting the existence of two clusters of the agent in the pig. However, it was not possible to determine whether the samples characterized in this study are related to distinct geographical regions or to any specific weather pattern, or are associated with any other variable, requiring further investigation to uncover such pending questions. The sequences found in the study corroborate with the ones also found by others, which relate different *Pneumocystis* sequences, in the same host such as man, primates, rats and bats.

Phylogenetic analysis...

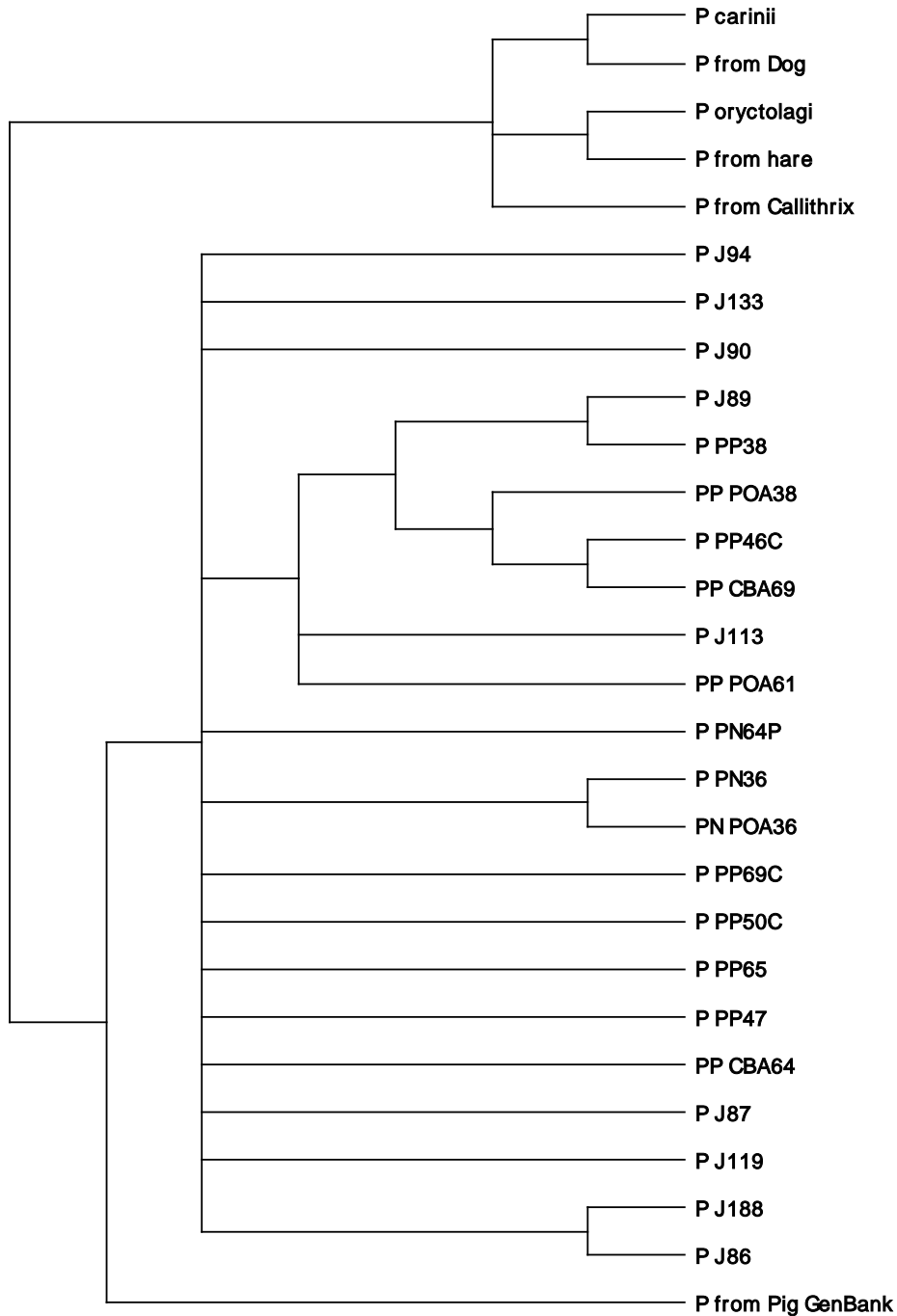


Figure 1. Phylogenetic relationships of *Pneumocystis* organisms from swine inferred from *mtLSU rRNA* sequences. The phylogram presented resulted from bootstrapped data sets obtained through parsimony analysis (heuristic search option in PAUP 4.0). The percentages above the branches are the frequencies with which a given branch appeared in 500 bootstrap replications. P PN: *Pneumocystis* from swine with normal lungs; P PP: *Pneumocystis* from swine of lungs with macroscopic pneumonia; C and CBA: samples from Cuiabá (MT); P and POA: samples from Porto Alegre (RS); P J: *Pneumocystis* from wild boar (*Sus scrofa*).

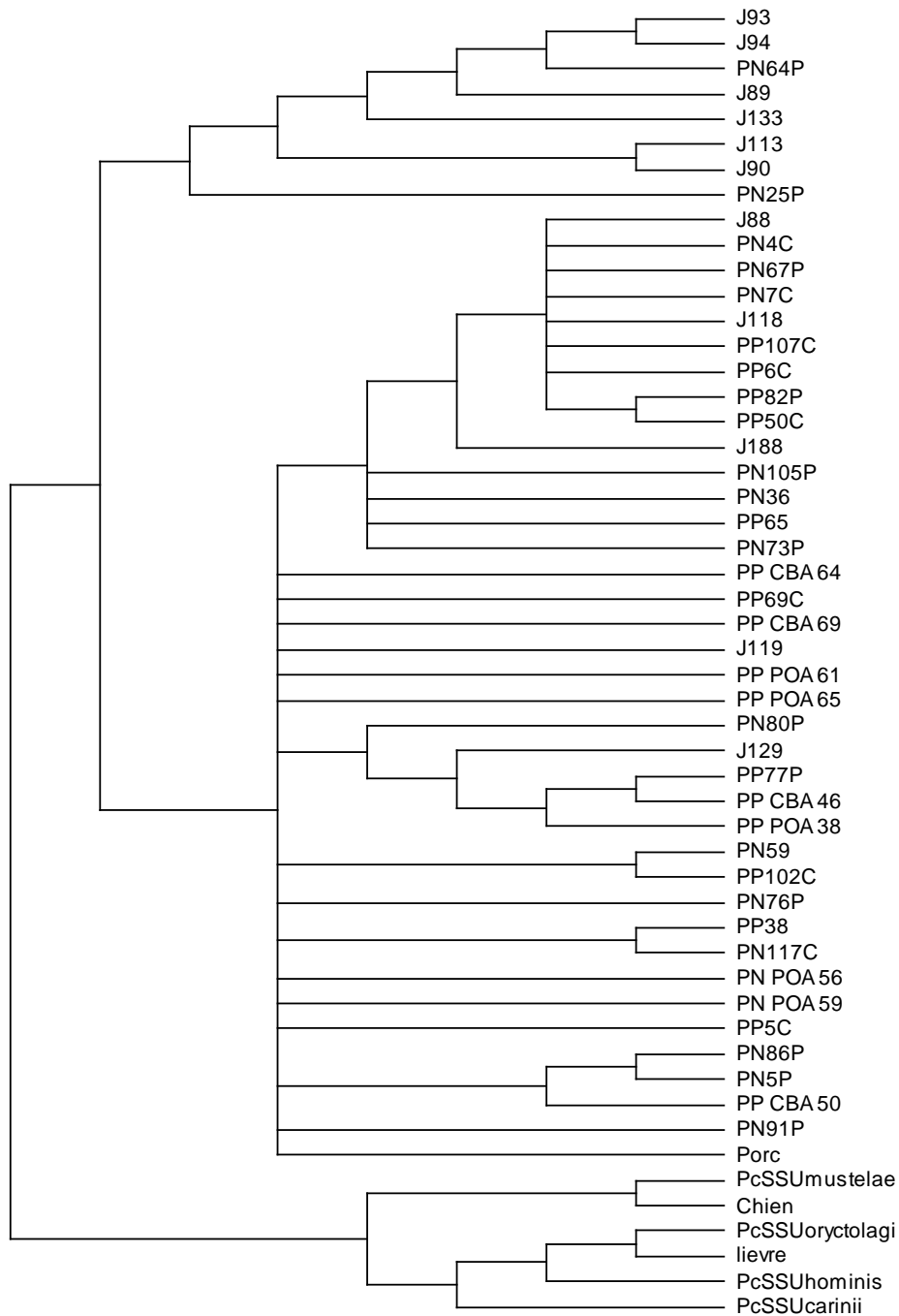


Figure 2. Phylogenetic relationships of *Pneumocystis* organisms from swine inferred from *mtSSU rRNA* sequences. The phylogram presented resulted from bootstrapped data sets obtained through parsimony analysis (heuristic search option in PAUP 4.0). The percentages above the branches are the frequencies with which a given branch appeared in 500 bootstrap replications. P PN: *Pneumocystis* from swine of normal lungs; P PP: *Pneumocystis* from swine of lungs with macroscopic pneumonia; C and CBA: samples from Cuiabá (MT); P and POA: samples from Porto Alegre (RS); P J: *Pneumocystis* from wild boar (*Sus scrofa*).

ACKNOWLEDGMENTS

The authors thank Marília T. O. Belmonte, Angela R. Belmontede Souza, Aline Schmitt, Clarissa Silveira Luiz Vaz, Laura de Almeida, Eliana Franco Lopes, Priscila Zlotowski, Ana O. Wallwitz, Roberto Medeiros, MAPA-SIPAMT, Judi M. Nóbrega, Plínio L. Lopes, and Antônio F. Barbosa Filho for their assistance. This work was supported by a grant (Processo 165 472409/2004-02) from the Conselho Nacional de Desenvolvimento Científico e Tecnológico 166 (CNPq), Brazil.

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