Study of the epidemiology of *Pneumocystis carinii* f. sp. *suis* in abattoir swine in Portugal

RITA ESGALHADO†, FRANCISCO ESTEVES*, FRANCISCO ANTUNES‡ & OLGA MATOS*

*Unidade de Parasitologia Médica, Grupo de Protozoários Oportunistas/VIH e Outros Protozoários – CMDT, Instituto de Higiene e Medica Tropical, Universidade Nova de Lisboa, †Faculdade de Medicina, Universidade de Lisboa, and ‡Clínica Universitária de Doenças Infecciosas, Faculdade de Medicina, Universidade de Lisboa, Hospital de Santa Maria, Lisboa, Portugal

*Pneumocystis* has been identified in various mammalian species, including domestic, wild and zoo animals. This study’s main objectives were: (1) to estimate the prevalence of the *Pneumocystis carinii* f. sp. *suis* infection in slaughtered pigs in Portugal, (2) assess the prevalence differences within distinct age groups of animals, (3) determine the possible associations between pulmonary lesions and the infection, and (4) genetically characterize the *P. carinii* f. sp. *suis* isolates recovered from infected animals using PCR with DNA sequencing. An epidemiological cross-sectional study was conducted using 215 pig lung tissue samples which demonstrated a global prevalence of 7% (14 positive samples). This value was later validated by statistical analysis as being representative of the national population prevalence. Regarding the assessment of relations between the different variables investigated during the study (age, gender, geographical region, type of farming, weight and pulmonary lesion) and the *P. carinii* f. sp. *suis* infection, no significant statistical differences were found, and apparently, no predisposing factors could be defined. Nevertheless, infection by *Pneumocystis* in pigs is ubiquitous and it can be detected in healthy animals. Thus, the colonization of *P. carinii* f. sp. *suis* among healthy individuals suggests that asymptomatic carriers can be an effective reservoir for susceptible animals and participate in the transmission of infection. The present data confirmed that porcine *Pneumocystis* is genetically distinct from *Pneumocystis* DNA detected in other mammalian hosts.

**Keywords** *Pneumocystis*, pigs, prevalence, genetic characterization

**Introduction**

*Pneumocystis* was first identified in the lungs of guinea pigs by Carlos Chagas (1909) and later in the lungs of rats by António Carini (1911). Since then *Pneumocystis* has been found in various mammalian species, including domestic, wild and zoo animals [1–8]. Although *Pneumocystis* can be responsible for pneumonia in immunocompromised mammals, the subclinical or latent infection is common [4,7,9]. Most diagnostic methods use lung tissue obtained at autopsies, although the bronchoalveolar lavage can also be employed for detection of *Pneumocystis* infection in animals [1,4,8,10,11].

Animal models have proven to be fundamental to the study of the *Pneumocystis* infection, both in epidemiological and comparative pathology terms [12–14]. Several of the animals studies (both in nature and laboratory) have demonstrated the antigenic and genetic diversity of the organisms infecting different mammals, leading to the plausible assumption that these variations may cause differences in the infectivity and pathogenicity of isolates [1,15]. A narrow *Pneumocystis* host specificity was demonstrated by cross-infection experiments. More recently, studies based on advanced molecular techniques have confirmed great genomic diversity among *Pneumocystis* isolated from different mammalian species. These observations support the theory that the genus *Pneumocystis* contains...
Pneumocystis carinii f. sp. suis pneumonia in pigs was originally described by Kucera in 1968 and by Seibold and Munnell in 1977 [2,16]. Since then, several studies have been conducted with this species which demonstrated the importance of this specific infection, which in turn raised interest in the role of this etiologic agent in animal health and its potential impact on pig meat production [8,9,14,15,17–19]. In countries like Denmark or Japan, the prevalence of the infection ranged from 7–60%, depending on geographic and farming factors, as well as the different diagnostic methods [2,3]. Studies conducted in Brazil in 2006 and 2007 by Sanches et al. [20,21], aimed to (1) correlate P. carinii f. sp. suis infection and porcine circovirus type II (PCV II) and (2) address P. carinii f. sp. suis infection in two regions of southern Brazil. The results of the first study indicated a 37% prevalence of P. carinii f. sp. suis and co-infection in 28% of the samples. There were no statistical significant correlations between the existence of lung lesions and infection by P. carinii f. sp. suis or PCV II [20]. On the other hand, in the second study, the presence of P. carinii f. sp. suis was statistically associated with the presence of pulmonary lesions and the prevalence of positive cases was 40% and 34% in pigs slaughtered in Rio Grande do Sul and Mato Grosso states, respectively [21]. More recently, in a study conducted in Southern Brazil in wild boars (Sus scrofa), P. carinii f. sp. suis was detected in 50%, PCV II in 37% and the concomitant presence of P. carinii f. sp. suis and PCV II was observed in 21% of the wild boars studied [22].

In Korea, Kim et al. studied the epidemiological characteristics of swine pulmonary infection caused by P. carinii f. sp. suis in Jeju Island. The P. carinii f. sp. suis infection prevalence found was 23% and porcine reproductive and respiratory syndrome virus (PRRSV) and porcine circovirus type 2 (PCV II) co-infection was a common phenomenon. This suggest that a P. carinii f. sp. suis infection may commonly follow primary viral infection in swine in Korea [23].

The main objectives of the present study were to determine the prevalence of P. carinii f. sp. suis in the Portuguese swine population, as well as to investigate the existence of associations between infection, different age groups and presence of pulmonary lesions. The role of diverse factors, such as sex, country region and type of farming, in the development of infection was also addressed.

Materials and methods

Animals and data

A total of 215 samples of pig (Sus domesticus) lungs were collected between July and September 2008 in slaughter-
Data analysis

The laboratorial results, as well as the sample profile and characteristics were subject to statistical analysis, using the Statistical Package for Social Sciences v.18.0 software (SPSS Inc., Chicago, IL, USA). To investigate associations between qualitative variables, such as group (healthy adults, adults with lung lesions and piglets), age, region, weight and sex, and infection by *P. carinii* f. sp. *suis*, the null hypothesis was tested using the nonparametric Chi-square test. The association between the weight (quantitative variable) and infection by *Pneumocystis* was also tested by formulating null hypothesis, in this case using the *t*-test (parametric approach). Additionally, the normal distribution was tested using the Kolmogorov-Smirnov and Levene’s tests. Statistical tests were applied to investigate associations at a significance level of 0.05.

Results

Of 215 pig lung samples analyzed in the present study, 14 (7%) were found to be positive by nested PCR. Table 1 summarizes sample profile and main features, both for the global and the positive sample pools.

Regarding the province of origin, 64% of the samples came from Lisbon and Tagus Valley, followed by Algarve (18%), Center and Alentejo (9%, each). The samples came mostly from intensive farming production (76%), the farms

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Table 1 Profile and characteristics of the samples of pig lungs collected.

<table>
<thead>
<tr>
<th>Analyzed samples (n) (%)</th>
<th>Positive samples (n) (%)</th>
<th>Within groups (%)</th>
</tr>
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<tbody>
<tr>
<td>Global sample 215 100</td>
<td>Global prevalence 14 7 100</td>
<td></td>
</tr>
<tr>
<td>Adult healthy pigs 105 49</td>
<td>Prevalence healthy adults 7 7 50</td>
<td></td>
</tr>
<tr>
<td>Adult pigs w/ lung lesions 51 24</td>
<td>Prevalence adults w/ lesions 3 6 21</td>
<td></td>
</tr>
<tr>
<td>Young pigs (piglets) 59 27</td>
<td>Prevalence piglets 4 7 29</td>
<td></td>
</tr>
<tr>
<td>Region</td>
<td>Region</td>
<td></td>
</tr>
<tr>
<td>Lisbon and Tagus Valley 137 64</td>
<td>Lisbon and Tagus Valley 6 4 43</td>
<td></td>
</tr>
<tr>
<td>Algarve 39 18</td>
<td>Algarve 5 13 36</td>
<td></td>
</tr>
<tr>
<td>Center 20 9</td>
<td>Center 2 10 14</td>
<td></td>
</tr>
<tr>
<td>Alentejo 19 9</td>
<td>Alentejo 1 5 7</td>
<td></td>
</tr>
<tr>
<td>Farming type</td>
<td>Farming type</td>
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<tr>
<td>Intensive 164 76</td>
<td>Intensive 9 5 64</td>
<td></td>
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<tr>
<td>Semi intensive 51 24</td>
<td>Semi intensive 5 10 36</td>
<td></td>
</tr>
<tr>
<td>Sex (adult pigs) *</td>
<td>Sex (adult pigs)</td>
<td></td>
</tr>
<tr>
<td>Male 92 59</td>
<td>Male 7 8 70</td>
<td></td>
</tr>
<tr>
<td>Female 62 40</td>
<td>Female 3 5 30</td>
<td></td>
</tr>
<tr>
<td>non-identified 2 1</td>
<td>non-identified 0 0 0</td>
<td></td>
</tr>
</tbody>
</table>

*Due to some of the slaughterhouses logistic procedures, it was impossible to obtain data for the sex and weight in the piglets group. Two of the adult pigs used in the sample were rejected for consumption, so it was also impossible to determine sex and weight for these two. All the samples were identified, stored and frozen until processing.
Pneumocystis in abattoir swine represented 24% of the sample. No samples from free-range animals were analyzed. Some 59% of the samples were from male and 40% female pigs. The average weight of the adult animals was 79.3 kg, the minimum recorded weight was 52.9 kg and the maximum was 103.9 kg (this represents a standard deviation of 10.1 and a variance of 101.3).

The overall prevalence of *P. carinii* f. sp. *suis* infection was 7%. When analyzing the results by groups, a prevalence of 7% was found in piglets and in healthy adults, with 6% of adults having pulmonary lesions. The 14 *P. carinii* f. sp. *suis* positive samples were distributed among four regions and eight different farms. The largest number of positive samples (six samples; 43%) were from the Lisbon and Tagus Valley, followed by the Algarve (five samples; 36%), Center (two samples; 14%) and Alentejo (one sample; 7%); 64% (nine positive samples) came from intensive farming and 36% (five samples) from farms with semi-intensive production systems.

The significance level found for all statistical tests was higher than 0.05, and as a result, no statistical significant differences were found among the groups of animals, their sex, weight, age, province, farming production systems and the infection. Therefore, no statistical evidence was found to secure the relationship between the variables studied and the *P. carinii* f. sp. *suis* infection.

The nested PCR products of the 14 amplified samples were successfully sequenced (in duplicate). The presence of nucleotide sequences analogous to the *P. carinii* f. sp. *suis* mtLSU rRNA gene, previously described by Wakefield et al., was confirmed in 13 isolates (93%) [33]. A novel *P. carinii* f. sp. *suis* mtLSU rRNA sequence was identified for the first time in one isolate (7%), in which a thymine was missing at position 84. This result was confirmed by repeating the nested PCR and the sequence analysis for this sample. The nucleotide sequences generated in this study were deposited in GenBank under accession numbers JN887823 and JN887824.

The relationships between the nucleotide sequences of *P. carinii* f. sp. *suis* mtLSU rRNA gene and the other *Pneumocystis* mtLSU rRNA sequences isolated from several host species were quantified applying the Neighbour Joining (NJ) method to the distance matrix (distances between all pairs of sequence from the multiple alignment). The generated data was used to construct a dendrogram (Fig. 1) [34,35]. The NJ dendrogram demonstrated that the *P. carinii* f. sp. *suis* sequences are clearly distinct from the other *Pneumocystis* sequences.

**Discussion**

To the best of our knowledge, the present report describes the first study in which the prevalence and genetic
characterization of *P. carinii* f. sp. *suis* in Portugal. The overall prevalence of infection in slaughter pigs was found to be 7%. Using StatCalc it is possible to verify with a 95% confidence level, that this prevalence is representative of the national population, considering a maximum error of 3%. This is consistent with the results published in 1991 by Settnes [2] in Denmark (prevalence of 7% – 119 samples), as well as with the results obtained by Shimizu in Hyogo province, Japan, (prevalence of 8% – 24 samples) [3]. However, the prevalence found in other studies conducted in Japan [18,19] and Brazil [20,21] were higher than those found in Portugal, suggesting that epidemiological factors such as geographic and climatic characteristics, and the specific productive/farming conditions, may influence the circulation and transmission of *P. carinii* f. sp. *suis* through the world.

The prevalence of *P. carinii* f. sp. *suis* was slightly higher in the piglets group, as expected, but we did not find a higher prevalence of infection in the animals with lung injury when compared to the healthy group. This could be due to the low overall prevalence recorded during the study and/or to the relatively small sample size analyzed. Previous studies showed that *Pneumocystis* is transmissible between hosts of the same species by the airborne route. [7,36]. *P. carinii* f. sp. *suis* colonization in apparently healthy animals suggests that these asymptomatic individuals may serve as an infective reservoir of *P. carinii* f. sp. *suis* for susceptible individuals, as occurs for other *Pneumocystis* infections in different hosts (e.g., *P. jirovecii* – humans). It is well established that immunocompetent hosts with subclinical *Pneumocystis* infection can transmit the infection by the airborne route to both susceptible and healthy individuals [6,7,36,37].

A recent study by Livingston and co-workers evaluated whether *P. carinii* infection in immunocompetent rats can cause idiopathic lung lesions previously attributed to rat respiratory virus (RRV). Cause and effect between *P. carinii* infection and the development of lung lesions was established in well controlled experiments, revealing a statistically significant association between lung lesion development and exposure to *P. carinii*. These data suggest that *P. carinii* infection in rats causes significant lung pathology that previously has been attributed to RRV [9]. Similarly, lung lesions in pigs are usually attributed to PCV II or even to PRRSV. Nevertheless, data from recent studies showed that *P. carinii* f. sp. *suis* infection is a common phenomenon and a potential etiologic agent that causes lung lesions in pigs [8,14,21]. The potential economic impact of this infection in pig meat production may be underestimated and neglected [9]. Further studies should be conducted in order to determine whether *P. carinii* f. sp. *suis* infection can cause idiopathic lung lesions previously attributed to other pathogens (e.g., PCV II) and to evaluate the implication of this infection in the economy of abattoir swine production.

Positive cases were recorded in all the analyzed regions, demonstrating the ubiquity of *P. carinii* f. sp. *suis* within continental Portugal territory [1]. Most of the positive samples came from intensive farming (64%), but, despite this, the prevalence of infection was higher in the semi-intensive system (five positive samples in 51; 10%), than in the intensive system (nine positive samples in 164; 6%). Less control of production variables and a less technical compliance in the semi-intensive regime could be the cause.

Despite no statistical associations being found between the studied parameters and the *P. carinii* f. sp. *suis* infection, it appears that there is a particularly higher prevalence of infection in the Algarve region (13%). In addition, both Algarve farms included in the study presented positive cases. These occurrences may be due to particular climatic conditions and/or to a less developed farming system in this specific region that leads to a higher prevalence of *P. carinii* f. sp. *suis* infection among the pigs for human consumption.

With regard to the molecular analysis, DNA amplification associated to nucleotide sequencing was found to be a effective robust, highly sensitive/specific methodology for detecting *P. carinii* f. sp. *suis* in pig lung samples. With exception of one isolate, the generated data indicated lack of sequence variation among the *P. carinii* f. sp. *suis* positive samples successfully analyzed at the mtLSU rRNA locus. This DNA sequence was aligned with the homologous sequences of *Pneumocystis* isolated from other host species, showing high divergence. The NJ dendrogram demonstrated that pig-derived *Pneumocystis* is genetically distinct from *Pneumocystis* DNA detected in other mammalian hosts, and the earlier suggested theory that *Pneumocystis* organisms are host specific thus seems justified [7,36,38].

In conclusion, infection by *P. carinii* f. sp. *suis* in pigs is apparently ubiquitous and it can be detected even in animals considered healthy. The detection of *P. carinii* f. sp. *suis* colonization among healthy individuals is an important epidemiologic issue since these animals can be an effective reservoir for susceptible animals and thus participate in the transmission of this infectious organism in livestock farms. The present data showed an overall prevalence of infection of 7% which suggests that *P. carinii* f. sp. *suis* infection must be evaluated in further studies in order to assess the evolution of the disease in Portugal and its impact in pig meat production. Additionally, preventive and therapeutic strategies should be adopted to control the incidence of *P. carinii* f. sp. *suis* infection and minimize potentially associated economic losses.

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