Molecular typing of *Pneumocystis jirovecii* found in formalin-fixed paraffin-embedded lung tissue sections from sudden infant death victims

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Previous studies have provided histological evidence of an association between primary *Pneumocystis* infection and sudden infant death syndrome (SIDS). The aim of this work was to determine the species of clustered *Pneumocystis* organisms found in formalin-fixed paraffin-embedded (FFPE) lung tissue sections from Chilean sudden infant death (SID) victims. This approach needed first to optimize a DNA extraction method from such histological sections. For that purpose, the QIAamp DNA Isolation from Paraffin-Embedded Tissue method (Qiagen) was first tested on FFPE lung tissue sections of immunosuppressed Wistar rats inoculated with rat-derived *Pneumocystis*. Successful DNA extraction was assessed by the amplification of a 346 bp fragment of the mitochondrial large subunit rRNA gene of the *Pneumocystis* species using a previously described PCR assay. PCR products were analysed by direct sequencing and sequences corresponding to *Pneumocystis carinii* were found in all the samples. This method was then applied to FFPE lung tissue sections from Chilean SID victims. *Pneumocystis jirovecii* was successfully identified in the three tested samples. In conclusion, an efficient protocol for isolating PCR-ready DNA from FFPE lung tissue sections was developed. It established that the *Pneumocystis* species found in the lungs of Chilean SID victims was *P. jirovecii*.

INTRODUCTION

Seroepidemiological data, obtained using several antigens, indicate that primary infection by *Pneumocystis* organisms is one of the most frequent infections in humans, affecting more than 90 % of normal healthy children during the first 2 years of life (Meuwissen *et al.*, 1977; Peglow *et al.*, 1990; Pifer *et al.*, 1978; Vargas *et al.*, 2001; Wakefield *et al.*, 1990a). However, only a small amount of information is available about the infection source and the clinical or pathological changes associated with this first contact with the fungus.

An association between *Pneumocystis* primary infection and sudden infant death syndrome (SIDS) has been reported in autopsy studies (Morgan *et al.*, 2001; Vargas *et al.*, 1999, 2001). *Pneumocystis* has also been reported in non-comparative studies in infants from Oxford, UK or Rochester and Yale, USA (Morgan *et al.*, 2001; Vargas *et al.*, 1999). Until now, the *Pneumocystis* organisms detected histologically in the lungs of sudden infant death (SID) victims have not been identified at the genomic level. This point could be important. On the one hand, a specific species of *Pneumocystis* could actually be involved in the aetiology of SIDS. On the other hand, if *Pneumocystis jirovecii* (Frenkel, 1999) was to be identified in such a context, the hypothesis of infants as reservoirs of *P. jirovecii* (Vargas *et al.*, 2001) would be strengthened.

Indeed, *Pneumocystis* infection is now recognized to be host-species-specific, suggesting that human *Pneumocystis* infection is not a zoonotic but an anthroponotic disease. Moreover, PCR studies have revealed that immuno-competent hosts develop transient *Pneumocystis* infections

and that they can transmit the parasite to susceptible hosts by an airborne route (Chabé *et al.*, 2004; Dumoulin *et al.*, 2000).

Thus, the aim of this study was to identify by molecular methods the species of clustered *Pneumocystis* organisms found in histological formalin-fixed paraffin-embedded (FFPE) lung tissue sections from Chilean SID victims. For that purpose, we had first to develop a DNA extraction method able to provide suitable DNA for a PCR assay. Therefore, the DNA extraction method was optimized on FFPE lung tissue sections from *Pneumocystis*-infected Wistar rats, before applying it to those from Chilean SID victims.

METHODS

Source of *Pneumocystis carinii* organisms. Corticosteroid-treated rats were used as the source of *P. carinii*. Wistar rats (Iffa-Credo, France) were given dexamethasone in their drinking water ($2 \ \text{mg} \ l^{-1}$) for 9–12 weeks (Dei-Cas *et al.*, 1998). Animals were housed in a conventional room of our laboratory animal facilities and were given standard food and water *ad libitum*.

Obtention and enumeration of *P. carinii* organisms. Parasites were separated from the lung tissue as described previously (Aviles *et al.*, 2000), with some modifications. Briefly, the lungs of infected animals were removed aseptically and cut into small pieces in sterile Dulbecco's modified Eagle's medium (DMEM). Parasites were extracted by agitation of the lung pieces with a magnetic stirrer for 40 min at 4 °C. The homogenate was poured through gauze and then filtered successively through 250 to 63 μm stainless-steel mesh. A polysucrose gradient (Histopaque-1077; Sigma Chemical), to obtain purified *Pneumocystis* organisms with a minimum of host contamination, was performed as follows. Polysucrose solution and parasite suspension in DMEM were prepared 1 : 1 (v/v) in a 15 ml tube and centrifuged at 1000 *g* for 15 min at 4 °C. The layer accumulated at the interface between DMEM and polysucrose solution was collected and washed twice with DMEM (2900 *g* for 10 min at 4 °C). After centrifugation, the pellet was resuspended in a buffered haemolytic solution, incubated for 10 min at 4 °C and centrifuged. The pellet was then resuspended in DMEM and centrifuged for 10 min at 4 °C. Parasite enumeration was performed on air-dried smears stained with toluidine blue O and RAL-555 as described previously (Aliouat *et al.*, 1993). Parasite samples from rat origin were stored in liquid nitrogen until utilization by using a cryo-preservation protocol described previously (ECA, 1996). This cryopreservation method has proved to be efficient in obtaining viable, infectious and ultrastructurally well-preserved *Pneumocystis* organisms (Durand-Joly *et al.*, 2002; ECA, 1996).

FFPE lung tissue sections from *P. carinii*-infected rats. The aim of this experiment was to obtain FFPE lung tissue sections from rats with different levels of infection by *P. carinii* that had the same features as those of the FFPE lung tissue sections from SID victims, i.e. FFPE 5 μm thick sections. Five 7-week-old female Wistar rats were administered dexamethasone ($2 \ \text{mg} \ \text{ml}^{-1}$) in drinking water from 15 to 20 days before inoculation until the end of the experiment. They were anaesthetized with a cocktail of drugs (150 mg ketamine hydrochloride kg^{-1}, 2·5 mg diazepam kg^{-1}, 0·75 mg atropine kg^{-1}) which was given intraperitoneally. Each animal was then inoculated non-surgically via the intratracheal route under sterile conditions with 20×10^6 *P. carinii* organisms suspended in 25 μl DMEM. Rats were killed sequentially at 1, 2, 4, 5 and 6 weeks after inoculation by an intraperitoneal injection of pentobarbital.

The chest cavity was opened and the lungs were removed aseptically. Six small pieces of lung were placed immediately into a 10 % buffered formalin solution. Fixed lung portions were ethanol-dehydrated and embedded in paraffin. Sections of 5 μm were then cut. About 20 slides per rat were made: 4–6 slides per rat were stained with Gomori Grocott silver methenamine stain for verifying the presence of the fungus in rats' lungs, and the remaining unstained slides were used for DNA extraction. The remaining, unfixed, fresh lung portions were used for *Pneumocystis* organisms' enumeration, as described above. One *P. carinii*-free Wistar rat was also killed and underwent the same handling.

Development of a de-paraffinization protocol. Dissolution of wax in xylene and ethanol, reversing the embedding and dehydration of tissue processing, was described by Goelz *et al.* (1985). In the present study, three de-paraffinization protocols, similar to Goelz's one, were performed on 5 μm thick FFPE lung sections from the Wistar rat killed at 5 weeks after inoculation with *P. carinii*. Protocol A consisted of dipping the slides containing the tissue sections in the following solutions for the specified times: LMR-SOL (Labo-Moderne) 5 min, twice; absolute ethanol 30 s; 95 % ethanol 30 s; 70 % ethanol 30 s; distilled water 30 s. Then, we scraped the tissue sections with a scalpel, and placed each one into a 2 ml microcentrifuge tube. LMR-SOL has similar properties to xylene but is less toxic and was used for these reasons in this work. Protocols B and C used the QIAamp DNA Isolation from Paraffin-Embedded Tissue method (Qiagen) using LMR-SOL or xylene, respectively. In contrast with protocol A, in protocols B and C, tissue sections were scraped with a scalpel and placed into a 2 ml microcentrifuge tube to proceed to de-paraffinization. For that, 1200 μl LMR-SOL or xylene was added to the tissue. The microcentrifuge tube was vortexed and centrifuged at 20 000 *g* for 5 min at room temperature. After removal of the supernatant, 1200 μl absolute ethanol was added to the pellet and mixed gently by vortexing. Then, the microcentrifuge tube was centrifuged at 20 000 *g*, the supernatant was removed and the ethanol wash was repeated. After that, the ethanol was carefully removed by pipetting and the open microcentrifuge tube was incubated at 37 °C for 10–15 min until total evaporation of the ethanol.

DNA extraction, PCR detection of *Pneumocystis* DNA and DNA sequence analysis. DNA was extracted from the de-paraffinized lung tissue sections in the microcentrifuge tubes using QIAamp DNA mini kit (Qiagen) according to the manufacturer's recommendations for the tissue protocol. PCR detection of *Pneumocystis* DNA was based on the method published by Wakefield *et al.* (1990b) using primers pAZ102-H and pAZ102-E, which amplified a portion of the mitochondrial large subunit (mt LSU) of the rRNA gene. Nested PCR with internal primers pAZ102-X and pAZ102-Y (Wakefield, 1996) was used when the number of *Pneumocystis* organisms in the samples was low and was insufficient to produce a visible band on an ethidium-bromide-stained gel after a single round of amplification. The reaction mixtures were prepared in 1× PCR buffer [75 mM Tris/HCl, pH 8·8, 20 mM $(\text{NH}_4)_2\text{SO}_4$, 0·01 % Tween 20] and contained, per 50 μl reaction, 3 mM of MgCl_2, 1 μM of both primers (Eurogentec), 400 μM of each dNTP, 1 U of Goldstar DNA polymerase (Eurogentec) and 20 μl of the purified DNA in the first round of amplification. In the second round, 2 μl of the first PCR product were used; the PCR conditions were the same as those of the first round. DNA amplification was carried out on a PTC 200 thermocycler. The conditions for the first- and second-round amplifications are as follows: denaturation at 94 °C for 1·5 min, annealing at 55 °C for 1·5 min and extension at 72 °C for 2 min for 40 cycles. The PCR product was analysed by electrophoresis in a 2 % agarose gel and was visualized after ethidium bromide staining. Negative controls with no added DNA were included in the DNA extraction step and in each amplification

run. Amplified PCR products were purified by filtration using microcon 50 and sequenced in both directions with a model ABI 377 automated sequencer by using an ABI Prism Dye Terminator cycle sequencing kit (Applied Biosystems), according to the manufacturer's instructions. The derived mt LSU rRNA gene sequences were compared with sequences available in the databases using the NCBI BLAST program.

Sensitivity of the DNA extraction and amplification from FFPE lung tissue sections. A DNA extraction followed by a single-round PCR amplification of the *Pneumocystis* mt LSU rRNA gene was performed on FFPE lung tissue sections from the five Wistar rats with different levels of *P. carinii* infection. To explore the sensitivity of the method, a nested PCR was carried out on the DNA extracted from 10 FFPE lung tissue sections from the Wistar rat with the lowest *P. carinii* rate.

FFPE lung tissue sections from Chilean SID victims. Four serially obtained histology sections of 5 μm thickness from FFPE lung tissue blocks were obtained for each of three 2- to 3-month-old Chilean infants who had died unexpectedly in the community and had an autopsy diagnosis of SIDS. Diagnosis of SIDS was based on the absence of symptoms prior to death, essentially negative macro- and microscopic autopsy and negative toxicological studies. One of the histology sections was used to identify *Pneumocystis* cysts by typical morphology using Gomori Grocott silver methenamine stain and optical microscopy. The other three FFPE sections were used in the study to evaluate the de-paraffinization protocol chosen after comparative assays using the rat model. Then, DNA was extracted, and the mt LSU rRNA gene sequences were amplified from each sample using *Pneumocystis*-specific primers and sequenced following the protocol described for rat FFPE tissue samples. Amplified sequences were compared with known *P. jirovecii* sequences.

RESULTS

Parasite counts in *Pneumocystis*-inoculated Wistar rats

The number of *Pneumocystis* total cysts increased from 2.1×10^5 to 2.0×10^7 in Wistar rats inoculated with *P. carinii* and killed at 1, 2, 4, 5 and 6 weeks after inoculation, respectively. In addition, tissue sections from the five rats showed *Pneumocystis* cysts using Gomori Grocott silver methenamine stain.

Selection of the de-paraffinization protocol using the *P. carinii*-infected Wistar rat model

The de-paraffinization methods were done on FFPE lung tissue sections from the Wistar rat killed 5 weeks after inoculation with *P. carinii*. The Qiagen de-paraffinization method with LMR-SOL (protocol B) was quickly discarded because no pellet was obtained in the microcentrifuge tubes. After DNA extraction from de-paraffinized lung tissue sections using protocols A or C followed by a single-round PCR assay, PCR products of approximately 346 bp in length were found on the ethidium-bromide-stained gel (data not shown). Direct sequencing identified rat-derived *P. carinii*.

As the Qiagen de-paraffinization method is performed in microtubes (see Methods), low quantities of reagent are

required. For this reason, protocol C was selected to perform de-paraffinization of FFPE lung tissue sections from Chilean SID victims.

Results and sensitivity of the DNA extraction and amplification from FFPE lung tissue sections

All five *P. carinii*-inoculated Wistar rats showed positive first-round PCR results (Fig. 1). The increasing intensity of the different bands obtained by PCR on the agarose gel after electrophoresis seems to be related to the number of *P. carinii* cysts found in the lungs of the Wistar rats (Fig. 1).
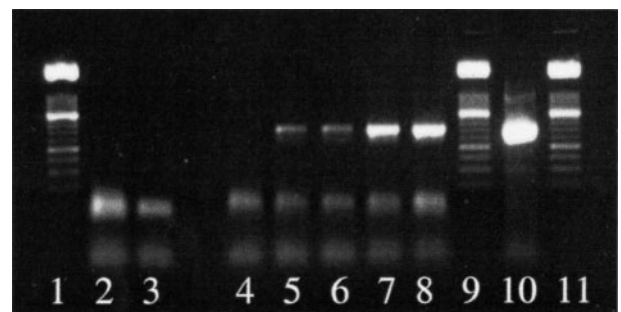
Six of 10 FFPE lung tissue sections from the Wistar rat with the lowest *P. carinii*-infection level (1 week after inoculation) showed positive first-round PCR results. However, all 10 samples showed positive second-round PCR results.

Therefore, protocol C was efficient for extracting PCR-ready *P. carinii* DNA from FFPE samples, even from an animal with a relatively low *P. carinii*-infection level (2.1×10^5 *Pneumocystis* total cysts).

Direct sequencing of all the PCR products obtained by either single-round PCR or nested PCR in the five infected Wistar rats identified *P. carinii* as the infecting organism. No PCR product was seen on the ethidium-bromide-stained gel for the non-infected Wistar rat, even after nested PCR.

FFPE lung tissue sections from Chilean SID victims

No PCR products were obtained in the first-round PCR performed on DNA extracted from the three FFPE lung

Fig. 1. Ethidium-bromide-stained agarose gel of the 346 bp products obtained after PCR amplification of DNA extracted from FFPE lung tissue sections from *P. carinii*-infected Wistar rats, killed at 1, 2, 4 and 6 weeks after inoculation with *P. carinii*. Lanes: 1, 9 and 11, DNA molecular mass marker XIII (Roche Diagnostics); 2 and 3, negative controls; 4, *P. carinii*-free Wistar rat; 5, 6, 7 and 8, *P. carinii*-infected Wistar rats killed at 1, 2, 4 and 6 weeks after inoculation with *P. carinii*, respectively; 10, positive control.
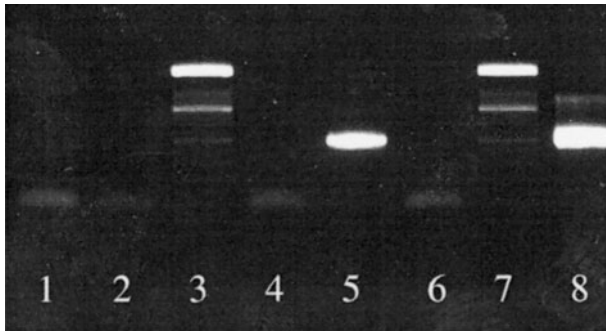
Fig. 2. DNA extracted from FFPE lung tissue sections from a SID victim, and results of a nested PCR assay targeting a portion of the *Pneumocystis* mt LSU rRNA gene. Lanes: 1 and 2, negative controls; 3 and 7, DNA molecular mass marker XIII; 4, 5 and 6, FFPE lung tissue sections from a *Pneumocystis*-infected infant (SID victim); 8, positive control.

tissue sections of each infant. However, a 260 bp product corresponding to the mt LSU rRNA locus was obtained in each infant in one of three histological sections amplified using nested PCR (Fig. 2). Direct sequencing identified *P. jirovecii* in the three infants. At the mt LSU rRNA locus of *P. jirovecii*, different sequence types have been described based on nucleotide polymorphism at positions 81, 85 and 248 [Sinclair *et al.* (1991), GenBank accession no. M58605; Lee *et al.* (1993), GenBank accession no. Z19053]. In this study, on the basis of a single-base polymorphism at position 85, we were able to determine two different genotypes of *P. jirovecii* among the three infants. Type C was observed in two infants and type A was observed in the third infant, according to the nomenclature system used by Sinclair *et al.* (1991).

DISCUSSION

Isolating high-quality genomic DNA from FFPE tissue can be difficult because only minimal quantities of intact DNA may be present in the sample. Indeed, while the routine fixative formalin preserves the tissue morphology, formalin fixation of tissues can cause the formation of protein–DNA cross-links, limiting the analysis of nucleic acids (Wu *et al.*, 2002). The amplification products can thus be reduced in quantity and size compared to fresh or frozen tissues (Greer *et al.*, 1991). Furthermore, the success of PCR from preserved tissue can vary with the type of fixative, fixation or storage time, temperature and PCR conditions. Isolating DNA from FFPE tissues can also be technically challenging with the presence of PCR inhibitors (Frank *et al.*, 1996).

Despite these problems, using the QIAamp DNA Isolation from Paraffin-Embedded Tissue method (Qiagen) using xylene (protocol C) associated with DNA extraction using the QIAamp DNA mini kit, we were able to obtain DNA from FFPE lung tissue sections suitable as a template for PCR. We successfully amplified a 346 bp portion of the mt

LSU rRNA gene of *Pneumocystis* in all the rat samples. In addition, the presence of several copies of this gene in the genome of *Pneumocystis* species ensures high sensitivity to this PCR detection assay as illustrated by the present results. PCR amplification of other molecular markers such as internal transcribed spacers (ITSs) or the dihydro-pteroate synthase (DHPS) gene has lower sensitivity than amplification of the mt LSU rRNA gene, probably because they are present in a single copy; however, the ITSs and the DHPS gene appear to be more informative regions for distinguishing isolates within the species *P. jirovecii* (Lane *et al.*, 1997; Tsolaki *et al.*, 1996, 1998).

Furthermore, direct sequencing of the PCR products allowed us to identify *P. jirovecii* in the lungs of three Chilean SID victims. These results reinforce the idea that infants could constitute an important part of the *P. jirovecii* reservoir (Durand-Joly *et al.*, 2003; Nevez *et al.*, 2001; Totet *et al.*, 2003a; Vargas *et al.*, 2001).

Although the mt LSU rRNA locus is usually used to identify *Pneumocystis* species, polymorphism at position 85 of the *Pneumocystis* mt LSU rRNA gene (Sinclair *et al.*, 1991; Wakefield, 1998) showed, in the present work, that presumably immunocompetent infants can harbour *P. jirovecii* genotypes similar to those found previously in immunosuppressed patients (Latouche *et al.*, 1997; Lee *et al.*, 1993; Wakefield *et al.*, 1994). Other molecular markers, such as ITS or DHPS sequences, are more informative at the infraspecific level of divergence. Recently, *P. jirovecii* was detected by molecular methods in asymptomatic infants or those presenting with a bronchiolitis episode (Nevez *et al.*, 2001; Totet *et al.*, 2001). ITS markers revealed a high diversity of *P. jirovecii* genotypes in infants, similar to those previously reported in immunosuppressed patients with *Pneumocystis* pneumonia (PCP) in Europe or the USA (Totet *et al.*, 2003a, b). The same results were found using DHPS markers (Totet *et al.*, 2001). The existence of similar genotypes among *P. jirovecii* isolates from different populations, such as asymptomatic infants, infants with bronchiolitis, SID victims, immunosuppressed adults or infants with PCP, supports the idea of an active circulation of *P. jirovecii* among humans. Individuals presenting different clinical forms of *P. jirovecii* infection could therefore constitute the reservoir of the fungus.

Sheldon (1959) highlighted the presence of small numbers of *Pneumocystis* organisms in the lungs of SID victims, and detected focal interstitial pneumonitis. He concluded that a subclinical form of *Pneumocystis* infection existed in apparently healthy infants or children. The implication that *Pneumocystis* organisms contribute to SIDS was suggested first by Vargas *et al.* (1999). In an autopsy study, these authors found clustered *Pneumocystis* organisms in 15 and 35 % of 27 English children and 134 Chilean children, respectively, all of whom were SID victims, against only 3 % of 342 infants who had died from other causes, at the hospital.

The technical knowledge acquired in the present work will allow us to develop a large-scale study to explore *P. jirovecii* genotypes in the lung tissues of SID victims. We also envisage being able to undertake an ultrastructural study targeting the identification of *Pneumocystis* life cycle stages in these tissues. This work is in progress in our laboratory.

Finally, archival FFPE tissue is an invaluable resource for molecular genetic studies. For example, in *Pneumocystis*-infection cases, the correlation of clinical manifestations or histopathological findings with specific molecular types of the species could be explored. As our procedure for DNA extraction from FFPE tissue sections is simple, and requires little time and handling (thus decreasing the possibility of contamination), it allows retrospective studies to be carried out on a large scale.

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