

## DETECTION OF *PNEUMOCYSTIS CARINII* IN LUNGS OF WILDLIFE MAMMALS FROM CROCKER RANGE PARK BY PCR AMPLIFICATION

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### ABSTRACT

*DNAs from 19 lungs of small wild mammals were screened for Pneumocystis carinii using PCR amplification. Products of PCR which could indicate the presence of P. carinii were seen in samples of DNA obtained from three different species. Since this is just a preliminary report, further sequencing is needed to verify the presence of P. carinii in these particular species.*

### INTRODUCTION

*Pneumocystis carinii* is an opportunistic pathogen capable of causing life-threatening pneumonia (PcP) in patients with AIDS and in other immunocompromised individuals [11]. Transmission of *P. carinii* is by airborne route. Apart from human, *P. carinii* is found in a wide variety of other mammalian hosts [10] worldwide. By using polymerase chain reaction (PCR) technique, *P. carinii* DNA has been detected in air samples [22], asymptomatic animals [12-15, 19], normal healthy individuals [17, 23] and immunocompetent patients with other diseases [16, 17] even if the organism was not detected by light microscopy.

It is generally believed that *P. carinii* is host species-specific since introduction of *P. carinii* from one host species into another host species did not result in discernible growth of *P. carinii* [1, 9]. This is consistent with the evidence showing that *P. carinii* isolated from different hosts are antigenically distinct [3, 4, 21] and phenotypically different [8, 14].

Recently more evidence demonstrated that the genome of *P. carinii* of one host differs markedly in sequence from the genomes of *P. carinii* from other hosts [20]. In addition, there is also variation in the chromosome and DNA sequence of *P. carinii* within a single host species (e.g., human, rat and ferret) [2, 7, 21]. For example, there are two genetically distinct varieties of the pathogen of *P. carinii* known to proliferate in the lungs of rats. These have been named 'prototype' (*P. carinii* f. sp. *cannii*) and 'variant' (*P. carinii* f. sp. *ratti*). The prototype is distinguished by the presence of a repeated DNA sequence (named RP3-1) and the 18S rRNA gene intron [7].

Despite intensive investigation in human and laboratory mammalian hosts, information on the occurrence and nature of infections in wild animals is still limited. Most studies on *P. carinii*, use laboratory animal models where infection is induced experimentally by administration of immunosuppressive drugs such as corticosteroids. For example, although *P. carinii* is commonly

found in laboratory rats or mice, for which very large numbers of animals have been examined, the nature of infection has not been detailed in wild rats. At the present time, studies on *P. carinii* from wild mammals have been done mainly in Europe (Czech Republic, Denmark, Finland) and in Asia (Japan) [5, 12, 28, 19]. It would be important to know whether *P. carinii* infecting wild mammals differ genetically from the strains found in laboratory counterparts.

This is the first report of detection of *P. carinii* in wildlife mammals in Malaysia. The DNA studied was isolated from the lungs of wildlife mammals caught at Crocker Range Park in Malaysia. Information on the occurrence of *P. carinii* in wild animals in Malaysia will provide information on the organism's distribution. Furthermore, sequencing *Pneumocystis* from wildlife will permit a more rigorous analysis of the evolutionary clock of *P. carinii*.

## MATERIALS AND METHODS

### Collection of Mammalian Lungs from Crocker Range Park

Mammalian lungs of Crocker Range Park were obtained from Dr. A.A. Tuen, University Malaysia Sarawak. Codes were used to refer the species of animals (Table 1). The animals were sacrificed with chloroform and the lungs were immediately excised and kept in liquid nitrogen prior to processing.

Table 1. ID Codes for the Crocker Range Park's mammals.

ID Code	Species	Common name
CRNP 1	<i>Cynopterus brachyotis</i>	Short-nosed fruit bat
CRNP 5	<i>Cynopterus brachyotis</i>	Short-nosed fruit bat
CRNP 7	<i>Megaerops ecaudatus</i>	Tailless fruit bat
CRNP 11	<i>Cynopterus horsfieldi</i>	Horsfield's fruit bat
CRNP 14	<i>Rattus rattus</i>	House rat
B1502	<i>Maxomys whiteheadi</i>	Whitehead's rat
CRNP 12	<i>Tupaia minor</i>	Lesser treeshrew
CRNP 15	<i>Maxomys whiteheadi</i>	Whitehead's rat
CRNP 16	<i>Maxomys whiteheadi</i>	Whitehead's rat
CRNP 17	<i>Tupaia minor</i>	Lesser treeshrew
CRNP 20	<i>Niviventer cremoriventer</i>	Dark-tailed tree rat
CRNP 22	<i>Tupaia Montana</i>	Mountain treeshrew
CRNP 23	<i>Tupaia Montana</i>	Mountain treeshrew
CRNP 26	<i>Leopoldamys sabanus</i>	Long-tailed giant rat
CRNP 25	<i>Sundasciurus brookei</i>	Brooke's squirrel
Mahua 1	<i>Tupaia Montana</i>	Mountain treeshrew
Mahua 2	<i>Maxomys?</i>	Rat?
Mahua 3	<i>Tupaia montana</i>	Mountain treeshrew
G. alab 1	<i>Maxomys suffer</i>	Red spiny rat

### Isolation of DNA from the Mammalian Lungs

DNA was isolated from the mammalian lungs using organic extraction and phase lock method at Universiti Malaysia Sarawak. The lung tissues were weighed and then minced in a petri dish.

The minced tissues were homogenized in Extraction buffer (100mg lung tissue/0.5ml Extraction buffer) containing 100mM Tris (pH 8.0), 100mM EDTA, 250mM NaCl, 1% Sarkosyl with proteinase K (100µg/ml) in 1.5ml Eppendorf tube. After digesting at 55°C for overnight, the homogenate was liquefied by gently pipetting with a disposable pipette. The homogenate was then sedimented for 10 min at 10,000g at 4°C. The viscous supernate was transferred to a fresh Eppendorf tube. Then, 250µl of the supernate was removed and put in another fresh Eppendorf tube and 250µl of 25:24:1 phenol:chloroform:isoamyl alcohol was added to it. The mixture was mixed gently but thoroughly and centrifuged at 14,000g for 1 min to obtain an aqueous phase.

The aqueous phase (top) was transferred to prespun phase lock tube and 250µl of 25:24:1 phenol:chloroform:isoamyl alcohol was added to it. The phase lock tube was used as it can ensure a clean separation between the organic and aqueous phase. The mixture was centrifuged at 14,000g for 1 min. The aqueous phase was measured again and transferred to Eppendorf tube and 1 volume of 7.5M of ammonium acetate was added. DNA in ammonium acetate was precipitated out using 2.5 volumes of 100% ethanol and spinning at 14,000g for 30 min at 4°C. Ethanol was removed from the DNA pellet and the DNA pellet was washed (no mixing) by adding 500µl of 70% ethanol and centrifuging at 14,000g for 5 min. Then, all ethanol was removed. The DNA pellet was air dried and resuspended in 50µl of Tris-EDTA buffer before shipping to the VA Medical Centre in Cincinnati Ohio for analysis.

The isolation of DNA was done twice to all except for one sample, CRNP-17. DNA was identified using the codes previously assigned to the animals (Table 2) with one exception. For code numbers with more than one tube, they were differentiated by the designation of either '1, or '2' (e.g. CRNP 1-1, CRNP 1-2). This system of coding was used throughout the experiments.

### PCR AMPLIFICATION

All PCR reactions were carried out in either a GeneAmp PCR System 2400 or 9700 thermocycler (PE Biosystems) using a HotStarTaq Master Kit (QIAGEN) and the indicated primer set. Conditions varied depending on the individual set of primers.

Table 1. ID Codes for the lungs DNA of Crocker Range Park's mammals.

ID Code	Species	ID Code	Species
CRNP 1-1	<i>Cynopterus brachyotis</i>	CRNP 20-1	<i>Niviventer cremoriventer</i>
CRNP 1-2		CRNP 20-2	
CRNP 5-1	<i>Cynopterus brachyotis</i>	CRNP 22-1	<i>Tupaia Montana</i>
CRNP 5-2		CRNP 22-2	
CRNP 7-1	<i>Megaerops ecaudatus</i>	CRNP 23-1	<i>Tupaia Montana</i>
CRNP 7-2		CRNP 23-2	
CRNP 11-1	<i>Cynopterus horsfieldi</i>	CRNP 26-1	<i>Leopoldamys sabanus</i>
CRNP 11-2		CRNP 26-2	
CRNP 14-1	<i>Rattus rattus</i>	CRNP 25-1	<i>Sundasciurus brookei</i>
CRNP 14-2		CRNP 25-2	

B 1502-1	<i>Maxomys whiteheadi</i>	Mahua 1-1	<i>Tupaia Montana</i>
B 1502-2		Mahua 1-2	
CRNP 12-1	<i>Tupaia minor</i>	Mahua 2-1	<i>Maxomys?</i>
CRNP 12-2		Mahua 2-2	
CRNP 15-1	<i>Maxomys whiteheadi</i>	Mahua 3-1	<i>Tupaia montana</i>
CRNP 15-2		Mahua 3-2	
CRNP 16-1	<i>Maxomys whiteheadi</i>	G.alab 1-1	<i>Mcxomys surifer</i>
CRNP 16-2		G.alab 1-2	
CRNP 17-1	<i>Tupaia minor</i>		

## RESULTS AND DISCUSSION

### 18s primers

The first sets of primers utilized were directed to the nuclear 18s ribosomal RNA subunit (18s). These primers are targeted to the nuclear small rRNA subunit and are capable of detecting three different products: *P. caninii* f.sp. *caninii* (prototype, 600bp), *P. caninii* f.sp. *ratti* (variant, 120bp), and mammalian host DNA (120bp). These primers are usually used during the initial PCR to make sure that there is DNA of sufficient quantity and quality present for subsequent PCR. A product was obtained from the majority of the reactions (Figure 1). All of the products were of the 120bp size, indicating that they could be either variant-like *P. carinii* or mammalian host DNA. While this did not provide any real information on what type, if any, of *P. carinii* may be present, it did indicate that the DNA present was of sufficient quantity and quality for further reactions.

Amplification of the products was not observed for some of the samples (Figure 1). It is possible that the DNA may have been denatured. Another more likely explanation is that there could be inhibitory factors in the DNA preparation such as hemoglobin since the DNA was isolated from the whole lung tissues. Since the stringency of the annealing was low (42°C) this may contribute to a lot of non-specific priming. This was shown by smears in most of the lanes (Figure 1). The smears could indicate that the target template was not present and instead the inappropriate template bind the primers and create non-specific products.

### Mitochondrial large subunit rRNA primers

The next set of primers used were pAZ-102H and pAZ-102E (H + E). These primers target the mitochondrial large subunit rRNA of *P. carinii* and produce a 346bp product. This set of primers was used because it has been shown to amplify *P. carinii* from a number of different species, including rats, rabbits, ferrets, and others. Only a few of the DNA (CRNP 1-2, CRNP 12-1, CRNP 16-1, CRNP 25-2) produced products that seemed to be of the proper size (Figure 2). The DNA that produced the proper sized products (CRNP 1-2, CRNP 12-1, CRNP 16-1, CRNP 25-2) was used in another reaction. For the second reaction, the undiluted DNA as well as a 1:10 dilution were used. No products were produced in this reaction (Figure 4). However, the negative controls of H and E primers also did not amplify correctly (Figures 2 and 4). Therefore, Repetition of experiment using the same primers should be done to address this problem.

### Mitochondrial small subunit rRNA primers

The final set of primers used were pAZI12-10F and pAZI12-10R (F + R). This set of primers targets the mitochondrial small subunit rRNA and produces products of varying sizes depending on the species of *P. caninii*. Known product sizes are 399bp for mouse and human, 423bp for rabbit, 445 for ferret, and 605 or 705bp for rat (variant and prototype respectively). Again, only a few products were seen from CRNP1-1, CRNP 7-1, CRNP 7-2, and CRNP 11-2 (Figure 3). However, in each there were bands of several different sizes. This was confirmed in several subsequent reactions (Figures 4 and 5). In these reactions, the DNA was amplified both undiluted as well as in a 1:10 dilution. The heaviest band was around the 700bp size, correlating with prototype *P. caninii*. In addition, another band approximately 1.2-1.3kb in size were seen in the product produced from CRNP 7-1 and 7-2. The products were run out on a gel and the ~.700bp band for the 1:10 dilutions of CRNP 1-1, CRNP 7-1, CRNP 7-2, and CRNP 11-2 were excised to be purified and sequenced. In addition, the 1.2-1.3kb band was also excised from the 1:10 dilutions of CRNP 7-1 and CRNP 7-2 for purification and sequencing.

The pronounced products in CRNP1-1; CRNP 7-1 and 2; and CRNP 11-1 and 2; (DNA obtained from *Cynoptyrus brachyotis*, *Megaerop ecaudatus* and *Cynoptyrus horsfieldi* respectively) using the F and R primers could indicate the presence of *Pneumocystis caninii* in this animals. Also, the presence of a band of approximately 1.2-1.3 kb could indicate that the existence of another variant of *Pneumocystis caninii* is possible. However, without doubt further purification and sequencing need to be done to verify these statements. Another matter that needs to be addressed and determined is how homologous these organisms DNAs will be to the primers that were used in the present studies. This is just a preliminary report on the samples that were obtained from Crocker Range Park. Further purification and sequencing are now underway in the Dr. Cushion's, Cincinnati lab.

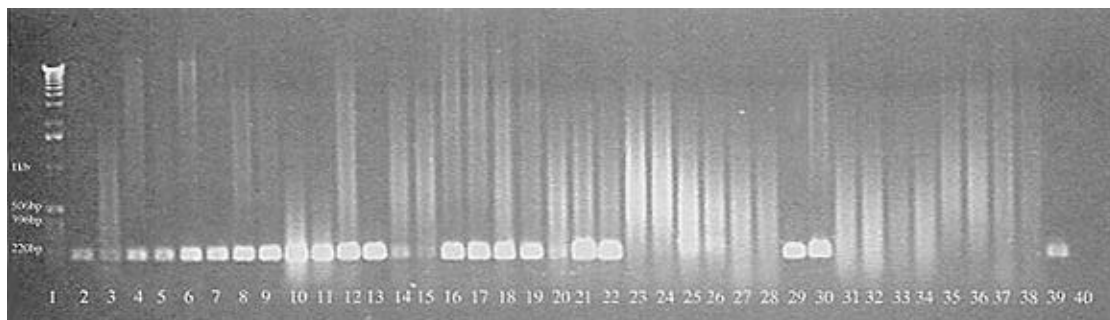


Figure 1. Amplification of Crocker Range Park's mammal DNA with 18s primers. Lane 1 is 1 kb ladder. Lanes 39 and 40 are the positive (purified rat *P. carinii*) and negative controls. Lanes 2-38 are the Malaysian mammals in the following order: CRNP 1-1; CRNP 1-2; CRNP 5-1; CRNP 5-2; CRNP 7-1; CRNP 7-2; CRNP 11-1; CRNP 11-2; CRNP 14-1; CRNP 14-2; B1502-1; B1501-2; CRNP 12-1; CRNP 12-2; CRNP 15-1; CRNP 15-2; CRNP 16-1; CRNP 16-2; CRNP 17-1; CRNP 20-1; CRNP 20-2; CRNP 22-1; CRNP 22-2; CRNP 23-1; CRNP 23-2; CRNP 26-1; CRNP 26-2; CRNP 25-1; CRNP 25-2; Mahua 1-1; Mahua 1-2; Mahua 2-1; Mahua 2-2; Mahua 3-1; Mahua 3-2; *G.alab* 1-1; *G. alab* 1-2. Product for either mammalian host or variant *P. carinii*

<http://www.arbec.com.my/pdf/art20julysep02.pdf>

(120 bp) was amplified in lanes 2-22, 25, 26, 29, and 30.

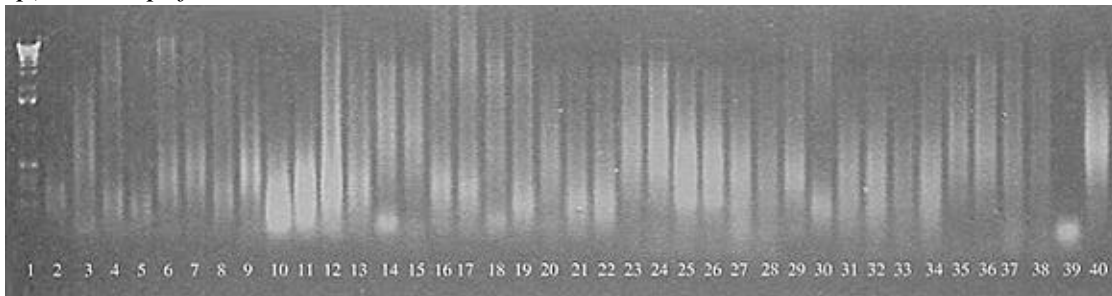


Figure 2. Amplification of Crocker Range Park's mammal DNA with H + E primers. Lane 1 is 1 kb ladder. Lanes 39 and 40 are the positive (purified rat *P. carinii*) and negative controls. Lanes 2-38 are the Malaysian mammals in the following order: CRNP 1-1; CRNP 1-2; CRNP 5-1; CRNP 5-2; CRNP 7-1; CRNP 7-2; CRNP 11-1; CRNP 11-2; CRNP 14-1; CRNP 14-2; B1502-1; B1501-2; CRNP 12-1; CRNP 12-2; CRNP 15-1; CRNP 15-2; CRNP 16-1; CRNP 16-2; CRNP 17-1; CRNP 20-1; CRNP 20-2; CRNP 22-1; CRNP 22-2; CRNP 23-1; CRNP 23-2; CRNP 26-1; CRNP 26-2; CRNP 25-1; CRNP 25-2; Mahua 1-1; Mahua 1-2; Mahua 2-1; Mahua 2-2; Mahua 3-1; Mahua 3-2; *G.alab* 1-1; *G. alab* 1-2. Amplification is seen for CRNP 1-2, CRNP 12-1, CRNP 16-1, and CRNP 25-2.

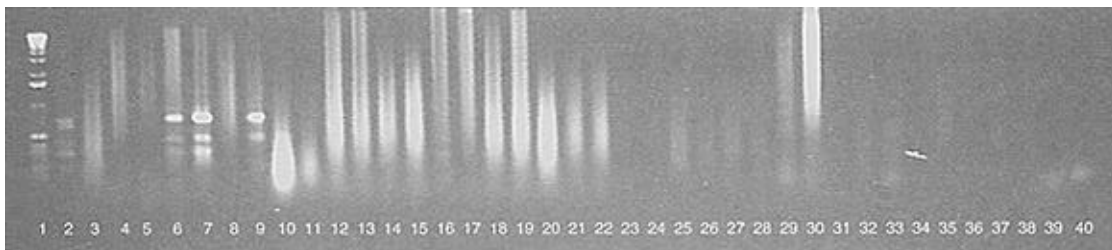
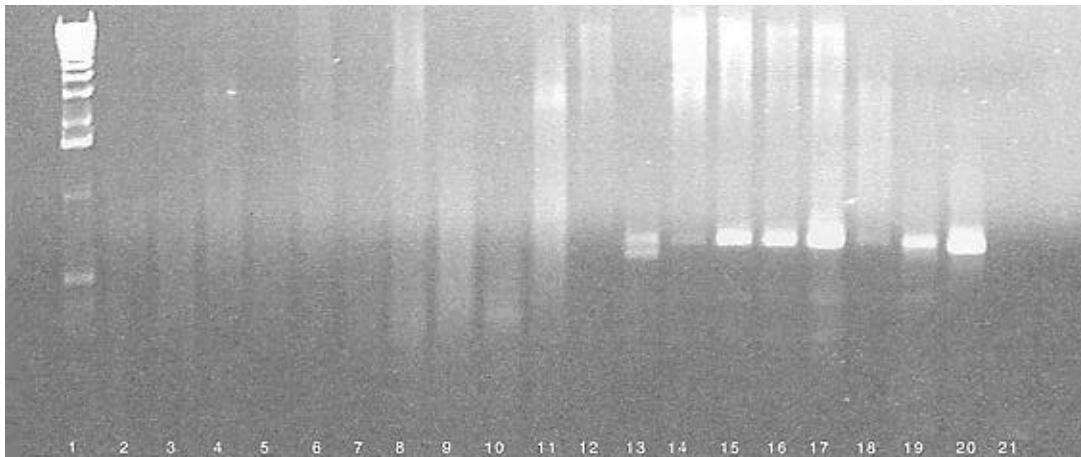
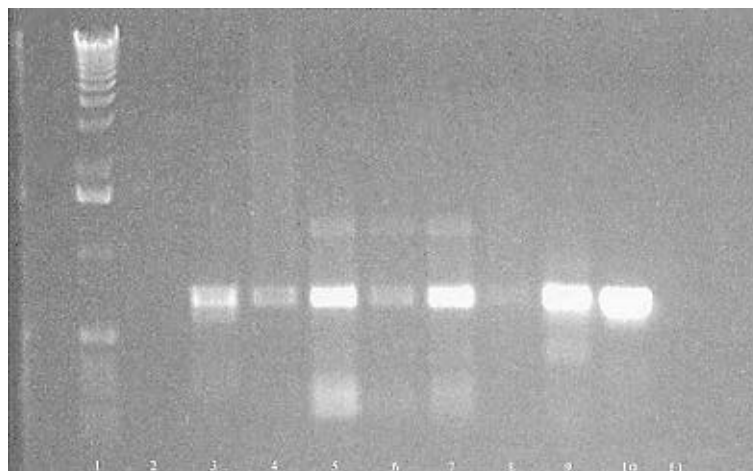


Figure 3. Amplification of Crocker Range Park's mammal DNA with F + R primers. Lane 1 is 1 kb ladder. Lanes 39 and 40 are the positive (purified rat *P. carinii*) and negative controls. Lanes 2-38 are the Malaysian mammals in the following order: CRNP 1-1; CRNP 1-2; CRNP 5-1; CRNP 5-2; CRNP 7-1; CRNP 7-2; CRNP 11-1; CRNP 11-2; CRNP 14-1; CRNP 14-2; B1502-1; B1501-2; CRNP 12-1; CRNP 12-2; CRNP 15-1; CRNP 15-2; CRNP 16-1; CRNP 16-2; CRNP 17-1; CRNP 20-1; CRNP 20-2; CRNP 22-1; CRNP 22-2; CRNP 23-1; CRNP 23-2; CRNP 26-1; CRNP 26-2; CRNP 25-1; CRNP 25-2; Mahua 1-1; Mahua 1-2; Mahua 2-1; Mahua 2-2; Mahua 3-1; Mahua 3-2; *G.alab* 1-1; *G. alab* 1-2. Amplification is seen for CRNP 1-1, CRNP 7-1, CRNP 7-2, and CRNP 11-2.



*Figure 4. Amplification of Crocker Range Park's mammal DNA with H + E and F + R primers. Lane 1 is 1 kb ladder. Lanes 2-11 are amplified with H + E and lanes 12-21 are amplified with F + R. Lanes 10 and 11 are the positive and negative controls for the H + E primers.:Lanes 20 and 21 are the positive and negative controls for the F + R primers. Lanes 2-9 are : CRNP 1-2, undiluted CRNP 1-2,1:10; CRNP 12-1, undiluted; CRNP 12-1, 1:10; CRNP 16-1, undiluted; CRNP 16-1, 1:10; CRNP 25-2, undiluted; CRNP 25-2, 1:10. Lanes 12-19 are: CRNP 1-1, undiluted CRNP 1-1,1:10; CRNP 7-1, undiluted; CRNP 7-1, 1:10; CRNP 7-2, 1:10; CRNP 11-2, undiluted; CRNP 1-1; CRNP 10-1; CRNP 7-1, undiluted; CRNP 7-1, 1:10; CRNP 7-2, undiluted; CRNP 7-2, 1:10; CRNP 11-2, undiluted; CRNP 11-2, 1:10.*



*Figure 5. Amplification of Crocker Range Park's mammal DNA with F + R primers. Lane 1 is 1 kb ladder. Lanes 10 and 11 are positive and negative controls. and negative controls for . Lanes 2-9 are : CRNP 1-1, undiluted CRNP 1-1,1:10; CRNP 7-1, undiluted; CRNP 7-1, 1:10; CRNP 7-2, undiluted; CRNP 7-2, 1:10; CRNP 11-2, undiluted; CRNP 11-2, 1:10.*

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