MycoSM Mycology Short Master:
LE INFEZIONI FUNGINE: UN PROBLEMA EMERGENTE DI SANITA' PUBBLICA, DALLA EZIOLOGIA ALLA TERAPIA

Pneumocystis species in mammals

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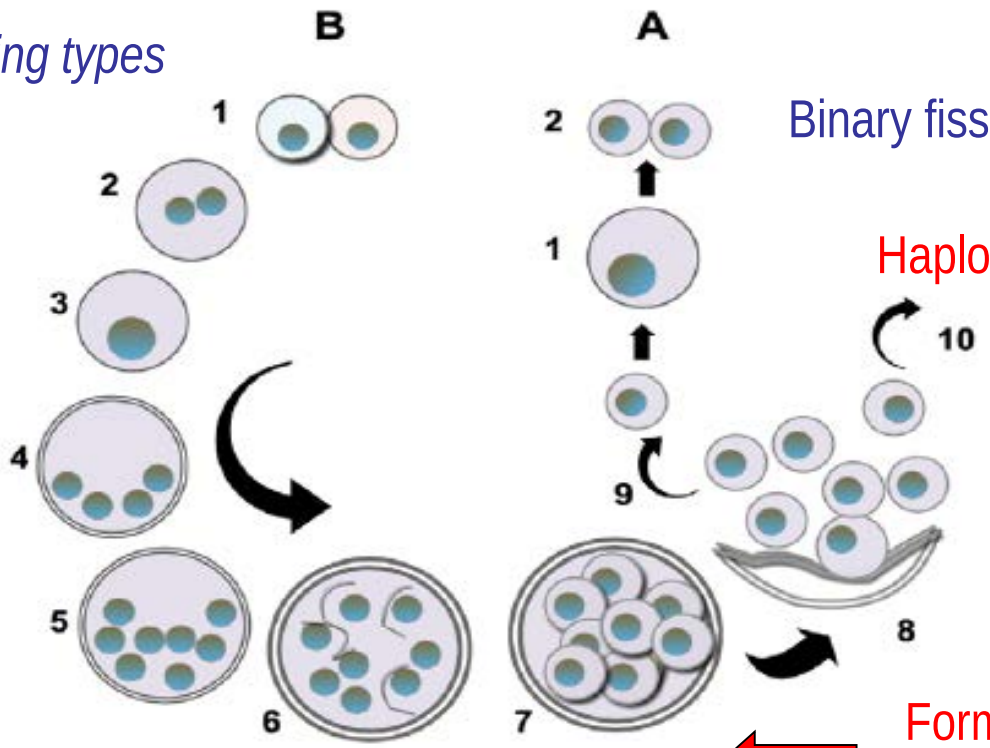
Introduction

- **Obligate** to live in low respiratory tract of host – extracellularly in the alveoli –Pneumocyte type I
- **Living host to survive** all stages of life cycle is required
- **Cannot** be maintained in continous **cultures**
- **Host specie-specific** - failures of experimental cross-species inoculation – it's not a Zoonosis
- Detected in the lungs of almost every mammal that has been evaluated for their presence
- Every mammals group has one or more Pneumocystis specie and/or sequence types (ST) most stronlgy associated

Putative life cycle of *Pneumocystis*

Sexual reproduction

Asexual phase



Cushion MT, 2010 Plos Pathogens

Usually trophic forms predominate over cyst forms by a ratio of ~10 to 20:1

Pneumocystis Pneumonia (PCP)

Life-treathing Pneumonia in Immunosuppressed individuals, HIV-infected infection, ect

- Common event in immunosuppressed laboratory animals (mice and rat)
- Rare event (or possible underdiagnosed) in companion and domestic animals

Pneumocystis species formally described

- ❑ *P. jirovecii* in humans
- ❑ *P. carinii* in brown rats
- ❑ *P. wakefieldiae* in brown rats
- ❑ *P. murina* in common mice
- ❑ *P. oryctolagi* in rabbits

- ✓ Trinomial system of special form (formae speciales) names associated with host genera
- ✓ nomenclature system recommended in 1994 by the Pneumocystis workshop

Pneumocystis carinii f.sp. "host genera"

P. carinii, P. murina and P. jirovecii the most extensively studied Pneumocystis species

Comparison of the mtDNAs of *P. carinii*, *P. murina*, and *P. jirovecii*

Characteristic	*P. carinii*	*P. murina*	*P. jirovecii*
Size (bp)	26,119	24,608	33,690–35,634[a]
Structure	Linear	Linear	Circular
G+C content (%)	29.8	29.8	25.7[b]
Noncoding region (%)	26.7	20.8	55.2[b]
Protein-coding genes (*n*)	14	14	14
rRNA genes (*n*)	2	2	2
tRNA genes (*n*)	25	28	25
rnpB RNA genes (*n*)	1	1	1
Orfs (*n*)[c]	4	2	1

[a]Four strains with 33,690, 35,517, 35,626, and 35,634 bp, respectively.
[b]Average of 4 genomes.
[c]Putative orfs with unknown functions.

Ma, Liang et al. "Sequencing and Characterization of the Complete Mitochondrial Genomes of Three Pneumocystis Species Provide New Insights into Divergence between Human and Rodent Pneumocystis." *The FASEB Journal* 27.5 (2013): 1962–1972. *PMC*. Web. 17 June 2018.

Nat Commun. 2016 Feb 22;7:10740. doi: 10.1038/ncomms10740.

Genome analysis of three Pneumocystis species reveals adaptation mechanisms to life exclusively in mammalian hosts.

Ma L[1], Chen Z[2], Huang da W[3], Kutty G[1], Ishihara M[4], Wang H[1], Abouelleil A[2], Bishop L[1], Davey E[1], Deng R[1], Deng X[1], Fan L[2], Fantoni G[1], Fitzgerald M[2], Gogineni E[1], Goldberg JM[2], Handley G[1], Hu X[3], Huber C[1], Jiao X[3], Jones K[3], Levin JZ[2], Liu Y[1], Macdonald P[2], Melnikov A[2], Raley C[3], Sassi M[1], Sherman BT[3], Song X[1], Sykes S[2], Tran B[3], Walsh L[1], Xia Y[1], Yang J[3], Young S[2], Zeng Q[2], Zheng X[3], Stephens R[3], Nusbaum C[2], Birren BW[2], Azadi P[4], Lempicki RA[3], Cuomo CA[2], Kovacs JA[1].

Pneumocystis Pneumonia (PCP) companion and domestic animals

- **Dogs**: around 50 case reports worldwide (Australia, USA and Europe) – mostly associated with breeds with suspected immunodepression genetically linked

- **Horses:** with breeds with suspected immunodepression genetically linked

- **Pigs:** intensive breeding systems (Europe and Brasil) – interstitial pneumonia moslty co-infections with respiratory virus and bacteria and *Mycoplasma pneumoniae*

- **Cats**: NO EVIDENCE OF SPUNTANEOUS PCP – also in cats receving prednisone and cyclosporine administration because of renal transplants for example


PcP and Pneumocystis in dog

Pneumocystis in dogs

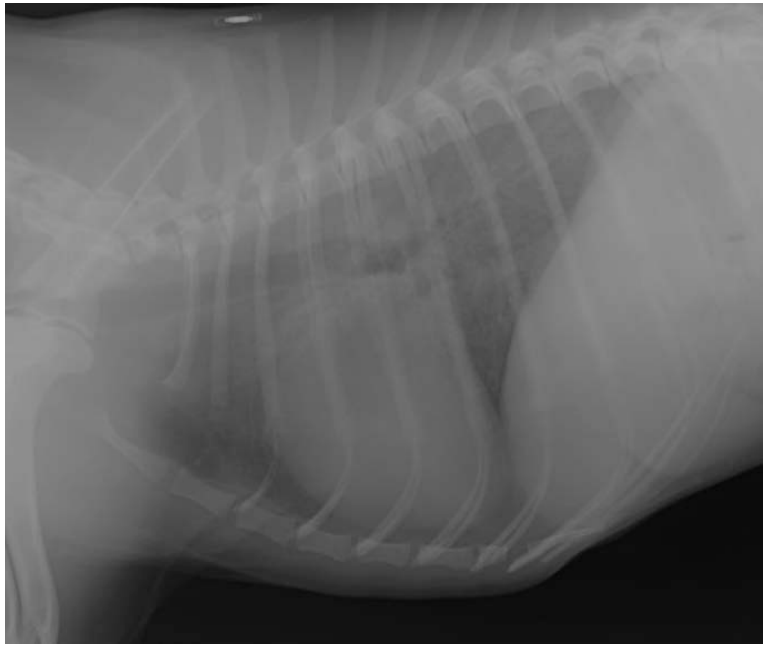
From 1974 to date – case reports in dogs ….

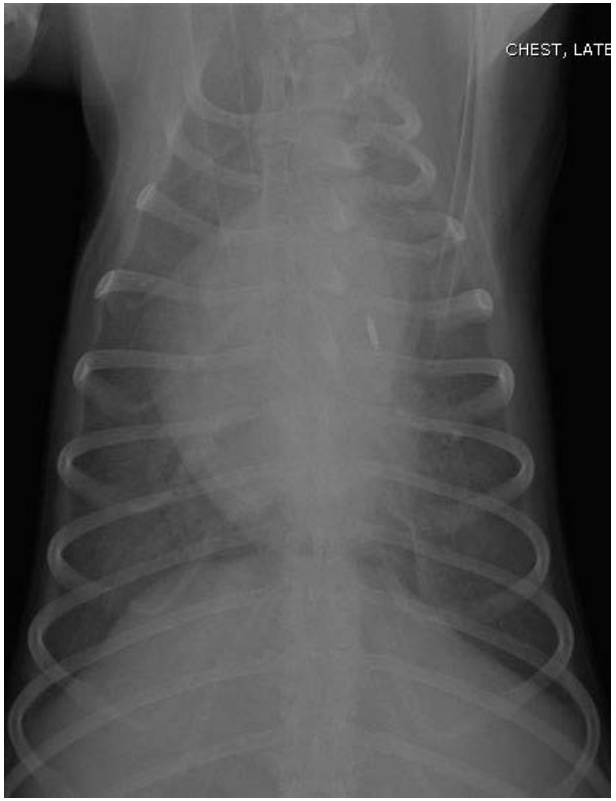
- Cavalier King Charles Spaniels (CKCS) 16
- Miniature Dachshunds 17
- Other breeds (for each 1 case described) 10

- dogs with predisposition for **impaired immunity (genetically)** are more likely to be affected
- presence of chronic coinfections, as well as a history of chronic therapy-resistant respiratory disorders
- NO specific clinical signs and/or haematologic abnormalities

Weissenbacher-Lang et al., 2018. Pneumocystosis in dogs: meta-analysis of 43 published cases including clinical signs, diagnostic procedures, and treatment. https://doi.org/10.1177/104063871774242

Canine PCP - interstitial fungal pneumonia





Clinical signs

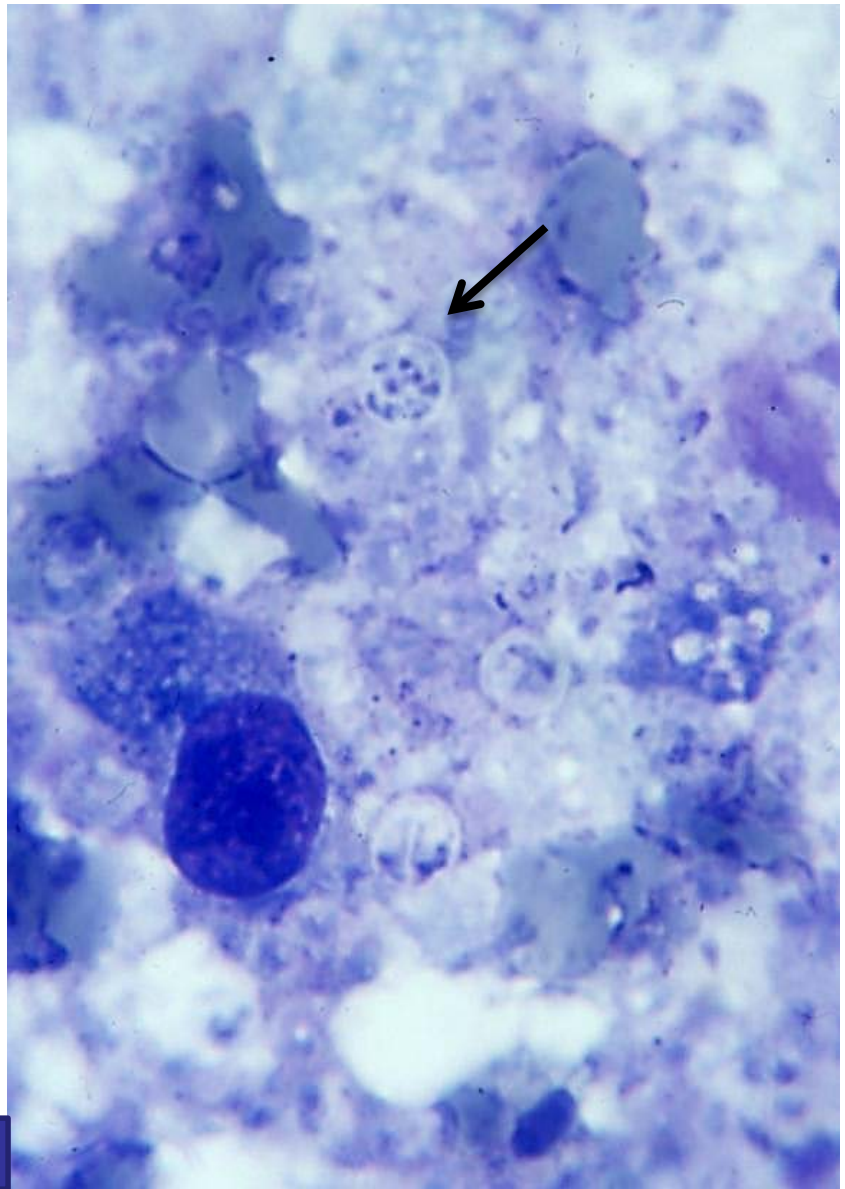
- respiratory signs, including laboured respiration and tachypnoea
- increased breath sounds on thoracic auscultation
- the presence of cough is variable
- thoracic radiographs reveal diffuse increased radiodensity of the pulmonary parenchyma

Diagnosis of PCP in dogs

- Identification of *P. f. sp. "canis* 'cysts' or "trophic forms" in bronchoalveolar lavage fluid (BAL)

- Cytology and histology

- PCR testing of BAL fluid or lung aspirates



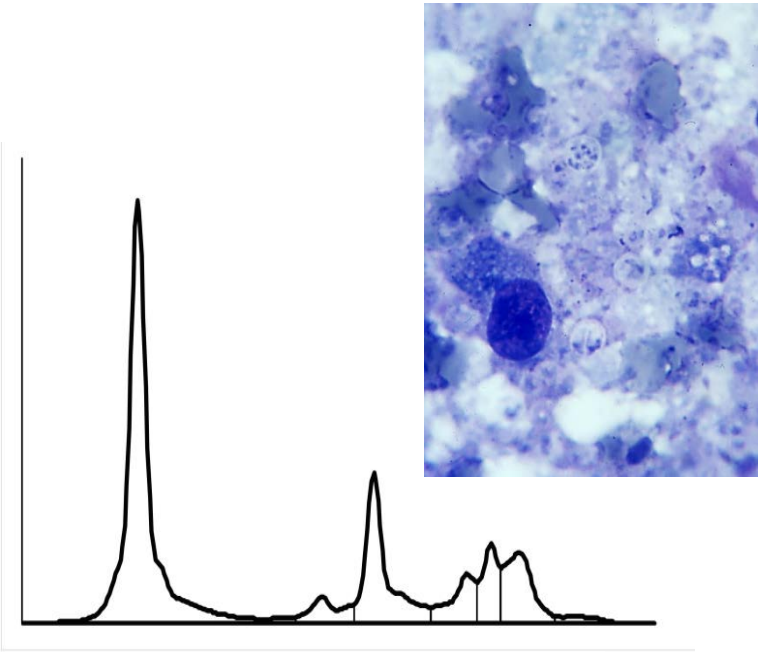
Courtesy Prof. Paul Canfield

Diagnosis can be challenging in a veterinary context

It is not uncommon that **a cytological diagnosis is not possible -** because specimens - deep bronchial washings or BAL fluid - contain so **few morphotypes** *Pneumocystis* even when the index of suspicion is high because

- of breed associations
- characteristic radiographic findings

accompanied by hypoxaemia.



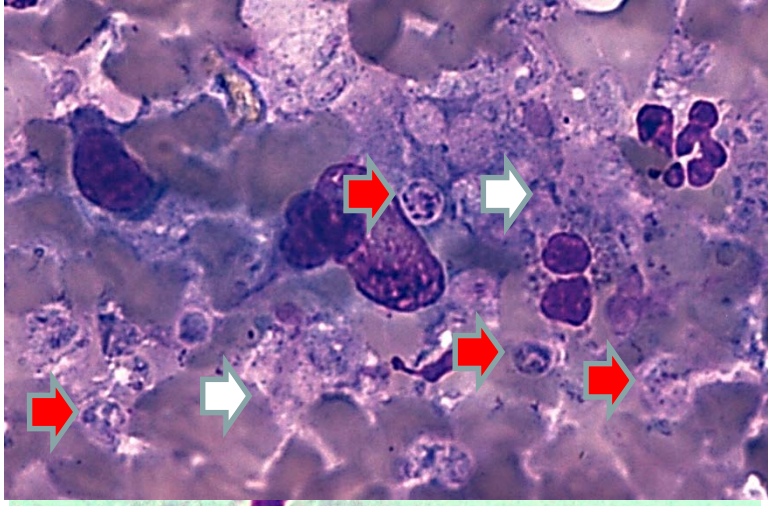
		min	max
Albumina (%) :	51.7	53.1	66.0
Globuline ALFA (%) :	22.1	11.7	17.3
Globuline ALFA1 (%) :	4.6	2.8	5.8
Globuline ALFA2 (%) :	17.5	7.9	13.0
Globuline BETA (%) :	24.7	10.2	20.1
Globuline BETA1 (%) :	6.8	1.9	3.9
Globuline BETA2 (%) :	6.9	3.1	5.8
Globuline BETA3 (%) :	11.0	6.0	11.3
Globuline GAMMA (%) :	1.5	6.4	14.5
Rapporto A/G (%) :	1.07	1.13	1.94

Electropherogram from PcP in a canine patient

Evidence of Pneumocystis morphotype 92 dogs tested

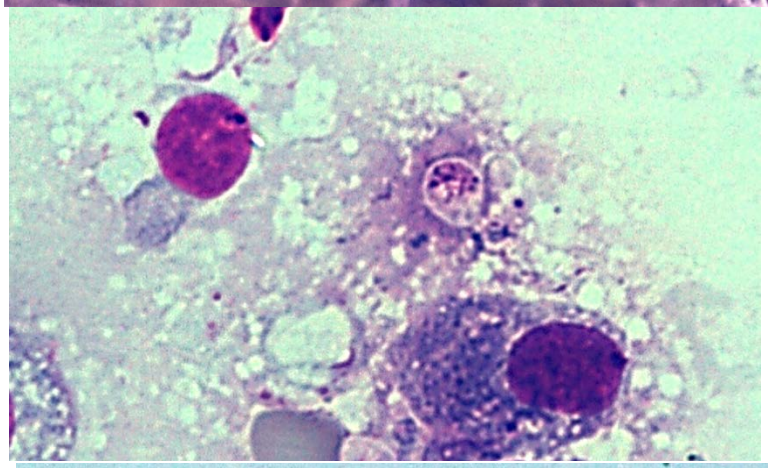
Group 1
dogs strongly suspected or confirmed of having PCP

10/16



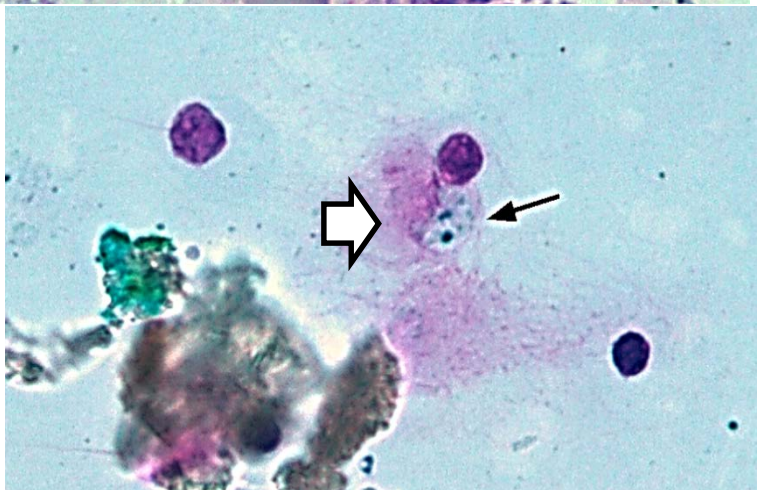
Group 2
dogs with non-PCP lower respiratory tract problems

0/65

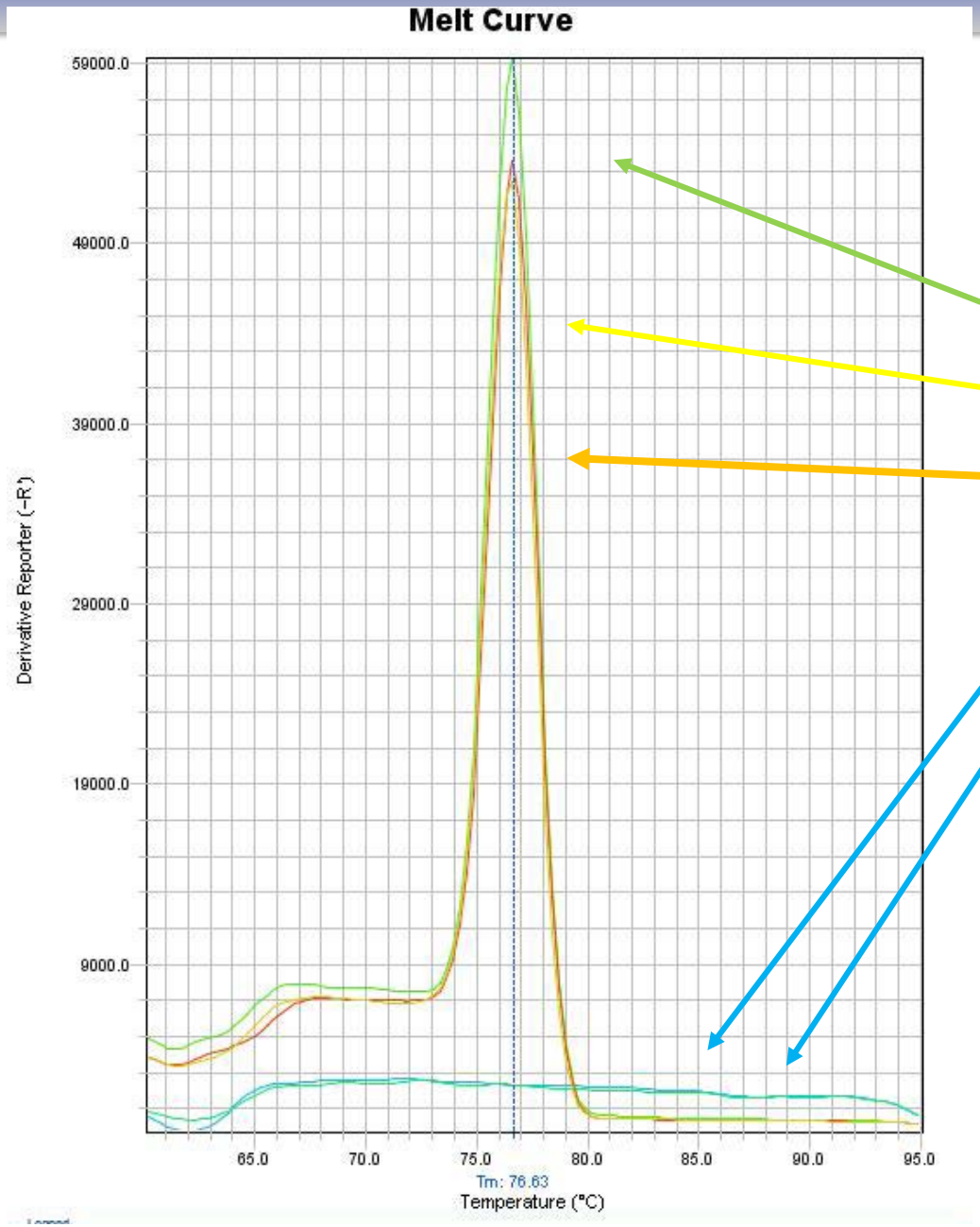


Group 3
dogs not suspected PCP

0/11



Molecular diagnosis of canine PCP



Sybr Green Real-time PCR targeting a 130-150 bp mtSSU rRNA amplicon

Sample 1
Sample 2
Positive control
NPC
NTC

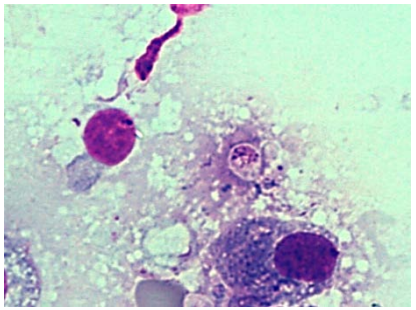
Organisms tested for specificity:
Cryptococcus laurentii,
Rhodotorula minuta,
Penicillium spp.,
Fusarium spp.,
Aspergillus fumigatus,
Aspergillus versicolor,
Emericella nidulans ,
Cryptococcus neoformans,
Candida albicans,
Bordetella bronchiseptica,
Pasteurella multocida

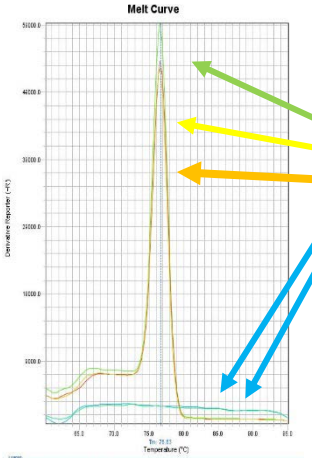
Comparison of Pneumocystis morphotype vs qPCR 92 dogs tested

› Med Mycol. 2017 Nov 1;55(8):828-842. doi: 10.1093/mmy/myx007.

Molecular diagnosis of Pneumocystis pneumonia in dogs

Patrizia Danesi [1], Silvia Ravagnan [1], Lynelle R Johnson [2], Tommaso Furlanello [3], Adelaide Milani [1], Patricia Martin [4], Susan Boyd [5], Matthew Best [6], Bradley Galgut [7], Peter Irwin [8], Paul J Canfield [9], Mark B Krockenberger [9], Catriona Halliday [10], Wieland Meyer [11], Richard Malik [12]





Sample 1
Sample 2
Positive control
NPC
NTC

Group 1
dogs **strongly suspected** or **confirmed** of having PCP

10/16

13/16

Group 2
dogs with **non-PCP lower respiratory** tract problems

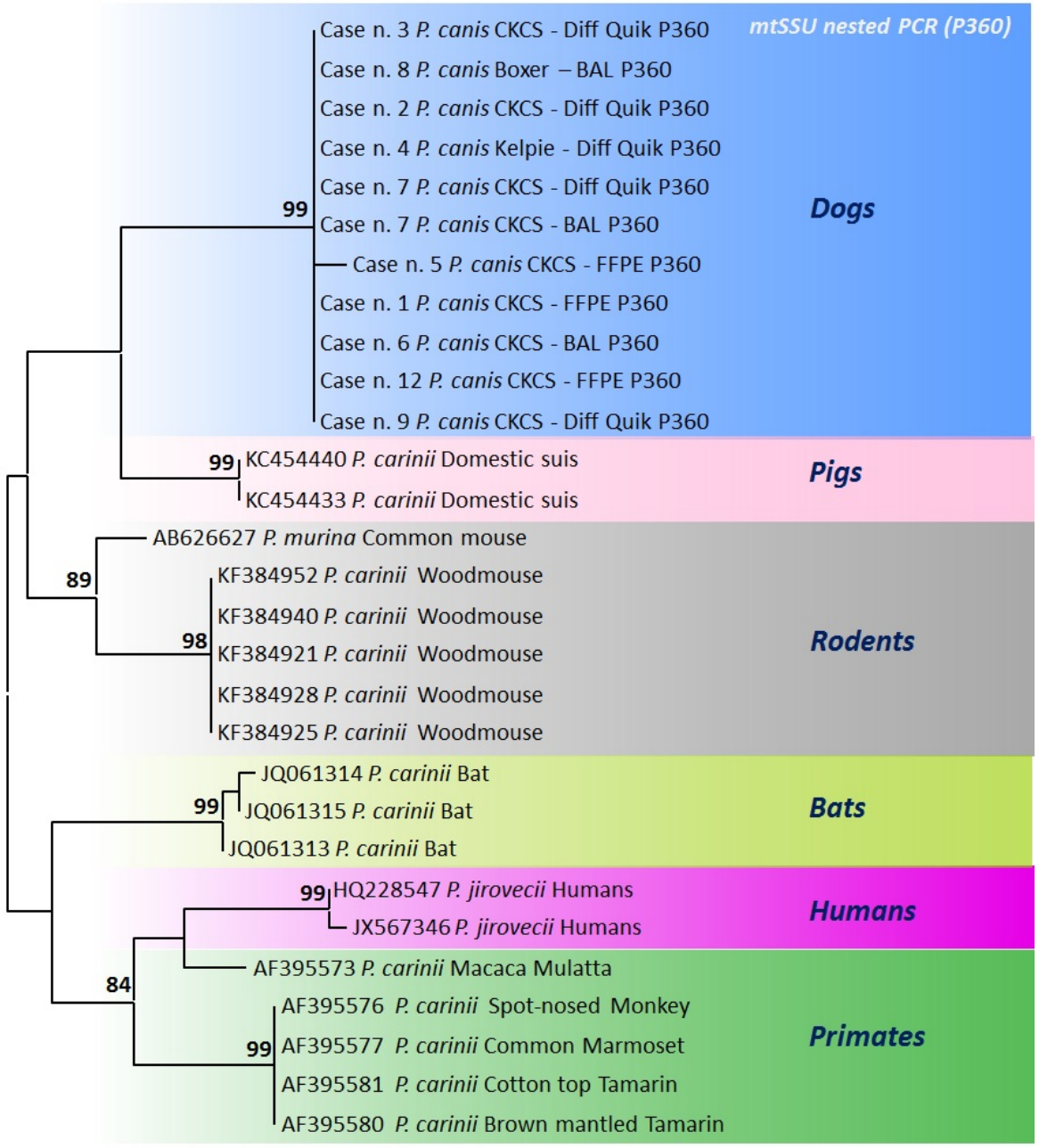
0/65

3/65

Group 3
dogs **not suspected** PCP

0/11

1/11



Muscle Alignment Maximum Likelihood method. 1000 Bootstrap replicates. The Tamura 3 parameter and G distribution was used as best model.

Pneumocystis Colonization in Dogs

			Breed	Number
Canine Cohort		255	Labrador Retreiver	14
Gender			CKCS	10
	Male	145	Pomeranian dog, German Sherperd	8
	Female	109	Dachshund, Yorkshire Terrier	7
	Unknown	1	Boxer, Chihuahua, French bulldog, Jack Russel Terrier	6
Age (year)			Beagle, American Staffordshire, Golden Retreiver, Zwergpinscher	5
	≤ 1	45	Weimaraner, Akita Inu, Bernese, Border collie, English Cocker Spaniel, English setter	4
	2-6	64	English Bulldog, Maltese, Pitt-bull, Rottweiler, Shih-tzu, Toy poddle, West Highland White terrier	3
	7-11	86	Vizla, Drahthaar, Bearded collie, Espagneul breton, Irish setter, Kurzhaar, Pug, Segugio italiano	2
	12-16	60	Affenpinscher, Afghan Hound, American Akita, Australian Shepherd, Belgian Shepherd, Bichon Frisè, Bolognese, Basset hound, Bracco italiano, Bullmastiff, Chow chow, Corso, Deutscher Pinscher, Giant Schnauzer, Great Dane, Greyhound, Italian greyhound, Japan Chin, Kooikerhondje, Leonberger, Maremma sheepdog, Samoiedo, Schnauzer, Segugio austriaco, Setter Gordon, Shiba-inu, Tibetan mastin, Vizla	1
Breed				
	Mongrel	70		
	Pure breed	185		

Danesi P., *et al*., 2022. Pneumocystis Colonization in Dogs Is as in Humans. Int J Environ Res Public Health. 2022. 8;19(6):3192. doi: 10.3390/ijerph19063192.

- Pneumocystis DNA in 22/255 dogs (8.8%) with lower airway or lung disease
- **no cyst or trophic forms of Pneumocystis were evident in any cytological preparations**
- Of the *P. canis*-qPCR positive dogs, 21 were purebreds, with a single crossbred dog.
- Boxer dogs (n = 3; 14%), CKCS (n = 2; 9%), Pomeranian (n = 2; 9%) and one each of various other breeds
- 16/22 (73%) of dogs were young (<1 year old), the remaining dogs 5 to 11 years old.
- 17 male dogs and 5 females
- Bacterial co-infections in 17/22 dogs (*Bordetella bronchiseptica* e/o *Mycoplasma pneumoniae)*
- Available haematological (n = 16) and biochemical (n = 14) measurements for the dogs showed that leukocytosis, hypoproteinaemia, and increased transaminases were the most common findings.

Molecular diagnosis of canine PCP

- **No guidelines exist in canine medicine** to define if PCP is present in an individual patient.
- To form a definitive diagnosis of PCP in a canine patient, some, ideally all, the following features need to be present:
- i. characteristic changes in chest radiographs, consisting of a dense and diffuse interstitial pattern, in association with right sided cardiac enlargement and signs of pulmonary hypertension on radiographs
- ii. extensive ground glass densities in pulmonary CT scans
- iii. the presence of cysts and/or trophozoite morphotypes on BALF cytology from stained smears (not always present but definitive when observed)
- iv. *P. canis* qPCR positivity, with CT less than 26 (our current arbitrary cut-off)
- v. favourable response to TMS therapy, usually with corticosteroids for the first few days of therapy

Molecular diagnosis of canine PCP

CASE REPORT | Full Access

Confirmed case of *Pneumocystis* pneumonia in a Maltese Terrier × Papillon dog being treated with toceranib phosphate

MP Best, SP Boyd, P Danesi

First published: 25 April 2019 | https://doi.org/10.1111/avj.12805 | Citations: 5

Case Reports > SAGE Open Med Case Rep. 2019 Apr 26;7:2050313X19841169.
doi: 10.1177/2050313X19841169. eCollection 2019.

Nested-polymerase chain reaction detection of *Pneumocystis carinii* f. sp. *canis* in a suspected immunocompromised Cavalier King Charles spaniel with multiple infections

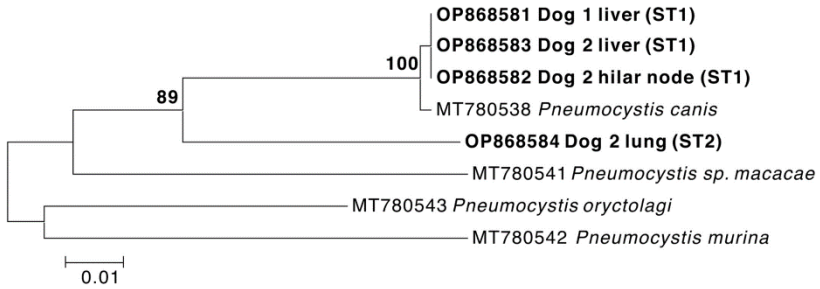
Matteo Petini [1], Tommaso Furlanello [2], Patrizia Danesi [3], Andrea Zoia [1]

CASE REPORT | Open Access

Oculosystemic pneumocystosis in 2 sibling Chihuahuas

Lynelle R. Johnson, Sean E. Hulsebosch, Austin K. Viall, Patrizia Danesi, Kevin D. Woolard, Sarah E. Cook, David J. Maggs, Brian C. Leonard

First published: 03 May 2023 | https://doi.org/10.1111/jvim.16729



Pneumocystis in companion and domestic animals: Cats

- First described in Mexico and Denmark in the period from 1950 to 1980 based on characteristic morphology of trophic forms and cysts (4% to 12%) (Davalos Mata and Varela 1959; Settnes and Hasselager 1984; Zavala and Rosado 1972)

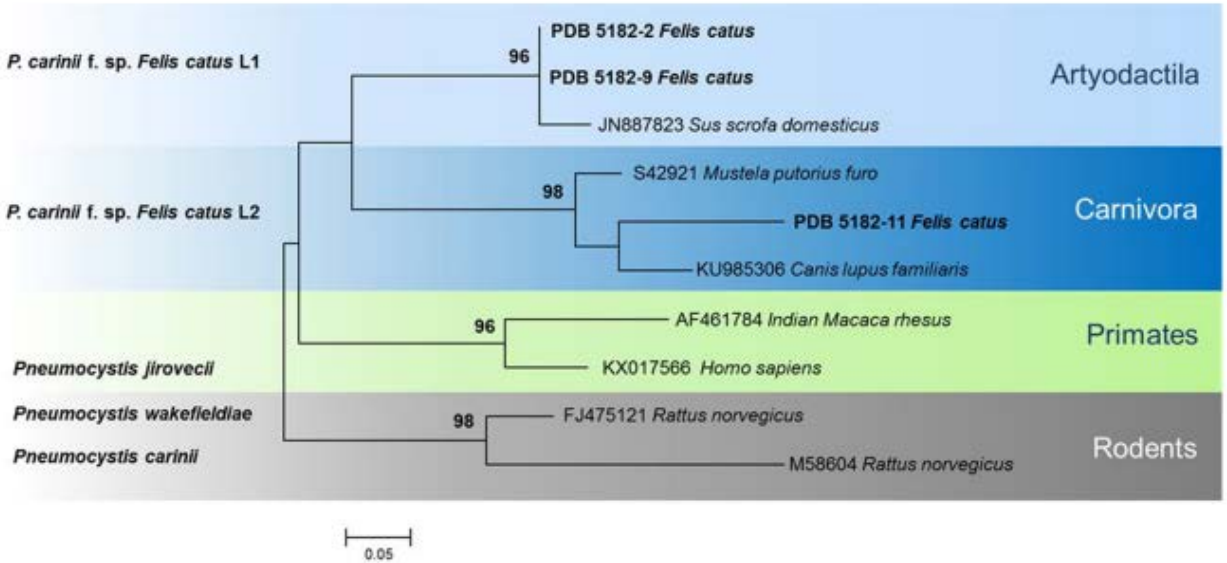
- No evidence of symptomatic PCP - considered to be colonized or infected subclinically

Animal models

- **FeLV positive cats never developed PCP (clinical, subclinical or colonisation)**
- Cats administered with high doses of corticosteroids and Pneumocystis mice inoculation developed PCP pneumonia (Cho et al. 1999; Shiota et al. 1990; Yuezhong & Baoping 1996)
- cats administered with Metil-prednisolone showed to be less susceptible to Pneumocystis infection when compared with rodents (Hong et al., 1992 Susceptibility of variuos animals to *P. carinii* infection. The Korean journal of Parasitology. 4:277-2819)
- **to date spontaneous or drug-induced PCP has not been described in the clinical feline literature, despite immunosuppression of cats by long-standing retrovirus infections or after kidney transplantation**.

Is Pneumocystis commensal organism in lungs of cats?

- Pulmonary tissue – feline necropsy (n=84)
- PCR on mtSSU and mt LSU – in house primers
- Pneumocystis DNA confirmed by sequencing in 24/84 (29%) cats
- Evidence of two different sequence types (or lineages)



INTERNATIONAL SOCIETY FOR HUMAN AND ANIMAL MYCOLOGY

Medical Mycology, 2019, 57, 813–824
doi: 10.1093/mmy/myy139
Advance Access Publication Date: 19 December 2018
Original Article

Original Article

Molecular detection of *Pneumocystis* in the lungs of cats

Patrizia Danesi[1,*], Michela Corrò[1], Christian Falcaro[1], Antonio Carminato[1], Tommaso Furlanello[2], Monia Cocchi[1], Mark B. Krockenberger[3], Wieland Meyer[4], Gioia Capelli[1] and Richard Malik[5]

Pneumocystis in companion and domestic animals: Cats

- Cats can be colonized or subclinically infected by Pneumocystis, without histological evidence of damage to the pulmonary parenchyma referable to pneumocystosis.

- Pneumocystis seems most likely an innocuous pathogen of cats' lungs, **but its possible role in the exacerbation of chronic pulmonary disorders or viral/bacterial coinfections should be considered further in a clinical setting**.

Colonization appears to occur naturally in both free-living and captive mammals

Phylogenetic relationships within the Pneumocystis genus mainly utilizing single-gene data sets

- PCR-based methods –and/or Sanger sequencing

- mtSSUrRNA - mitochondrial small subunit of rRNA
- mtLSUrRNA - mitochondrial large subunit of rRNA
- DHPS - dihydropteroate synthetase
- ITS 1/2 – Inter-trascribed spacers of rRNA

1. Group of highly diversified species
2. High host species-specificity of with mammalian groups
3. Pneumocystis species and/sequencetypes co-infection

How many Pneumocystis species and/or sequencetypes in mammals?

Single mammalian specie

At least as much as the mammals group investigated

Hundreds of mammalian species

Pneumocystis species in mammals

Commensals

Opportunists

Pathogens

Pneumocystis has mostly a “commensal like”life style
In immunologically intact mammals → little to no pathogenic effects

Losing of some immune function of the host (by disease or chemotherapeutic agents), the organisms can take advantage as would be an opportunist

Enter into a more aggressive state which in some cases can be associated with clinical symptoms

Asymptomatic carriers – high prevalence

Order	Family	Species	Prevalence	Diagnostic tool	Country
Rodentia	Murinae	*Apodemus syilvaticus*	67%	PCR	France/Spain
		Apodemus syilvaticus	71%	PCR	Spain
		Apodemus syilvaticus	55%	PCR	France
		Apodemus syilvaticus	12.5%	Microsopic	Denimark
	Arvicolinae	*Mycrotus agrestis*	17%	Microsopic	Finland
Insectivora	Sorcinae	*Sorex araneus*	70%	Microsopic	Finland
		Sorex caecutiens	17%	Microsopic	Finland
		Notiosorex crawfordi	34	PCR	California
		Sorex ornatus	13	PCR	California

Asymptomatic carriers – low parasite rate

- Most animals appear to be colonized by *Pneumocystis* by **low parasite rates** (low number of Pneumocystis morphotype with no lung pathology/alteration)
- Frequently co-infected with **more than one strain**

DOI: 10.1111/j.1550-7408.2009.00465.x

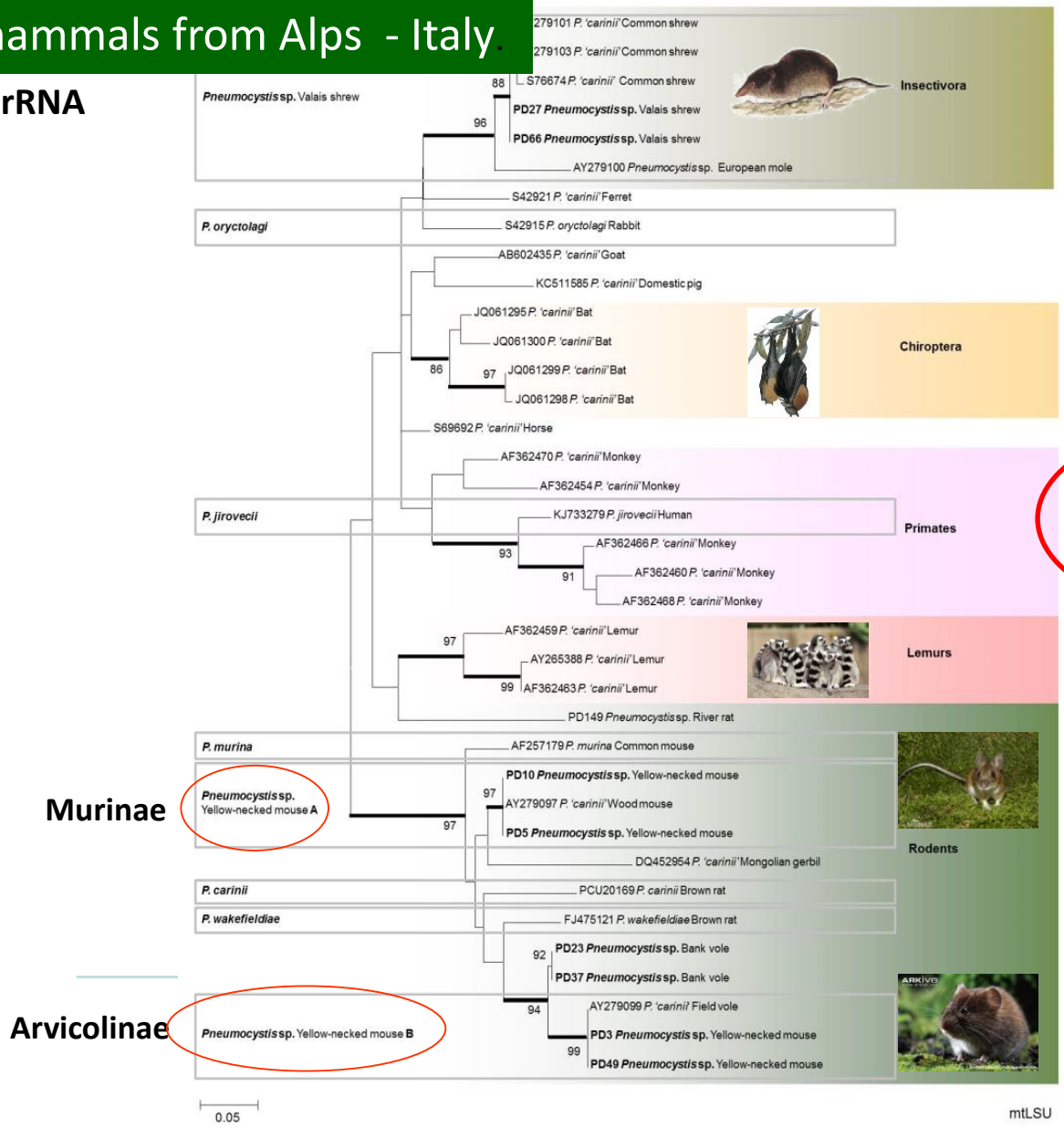
***Pneumocystis carinii* and *Pneumocystis wakefieldiae* in Wild *Rattus norvegicus* Trapped in Thailand**

MAGALI CHABÉ,[a,b] VINCENT HERBRETEAU,[c] JEAN-PIERRE HUGOT,[d] NOEMI BOUZARD,[e] LUCIE DERUYTER,[e]

Rodent Pneumocystis host specificity **is mostly limited to the generic level**

Wild small mammals from Alps - Italy.

mtLSU rRNA



Murinae

Arvicolinae

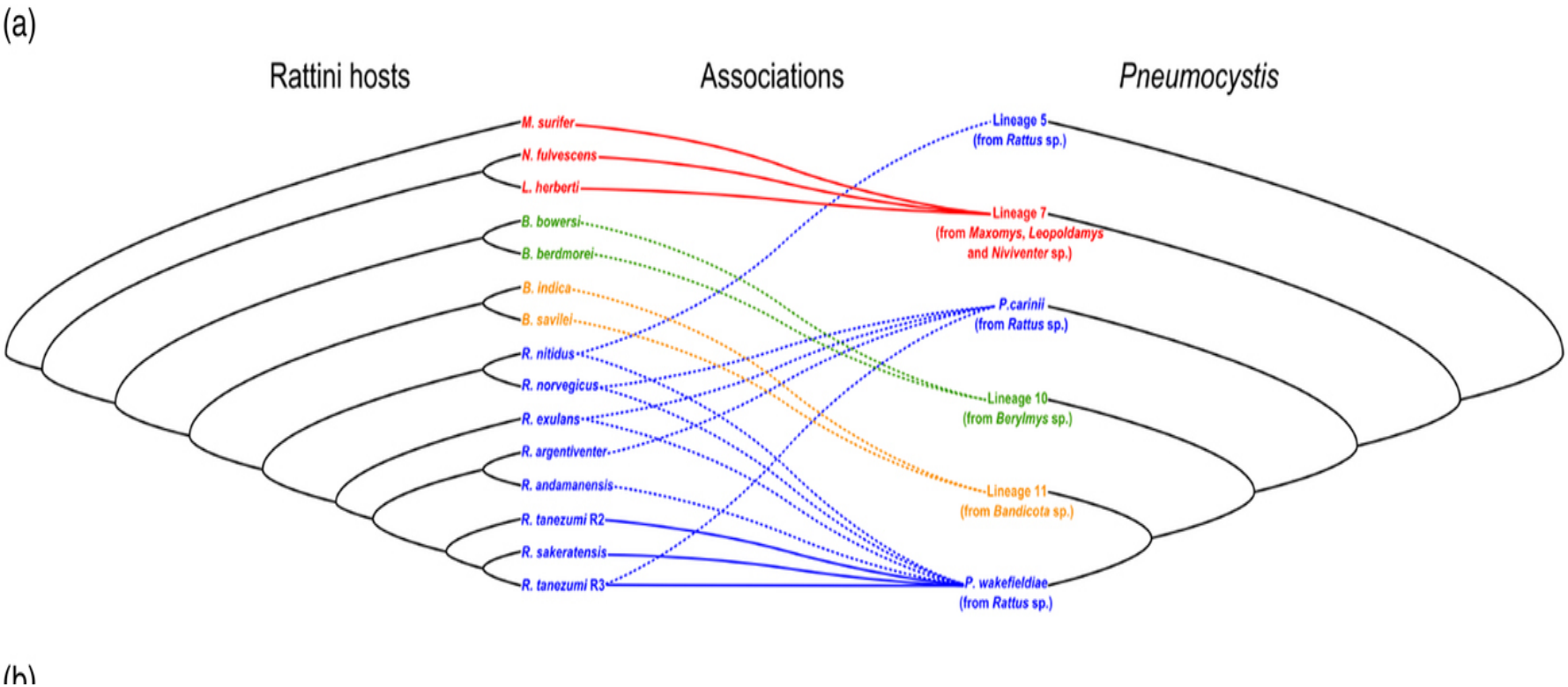
Shrew **79%**

Yellow necked-mouse **27%**

Bank-vole **67%**

Danesi *et al.*, 2016. Barcoding markers for Pneumocystis species in wildlife**.** Fungal Biol. 120(2):191-206.

Pneumocystis in small mammals



Murid rodent Pneumocystis host specificity **is mostly limited to the generic level rather than the species level as sequence types are shared among several host species several mtLSU rRNA and mtSSU rRNA**

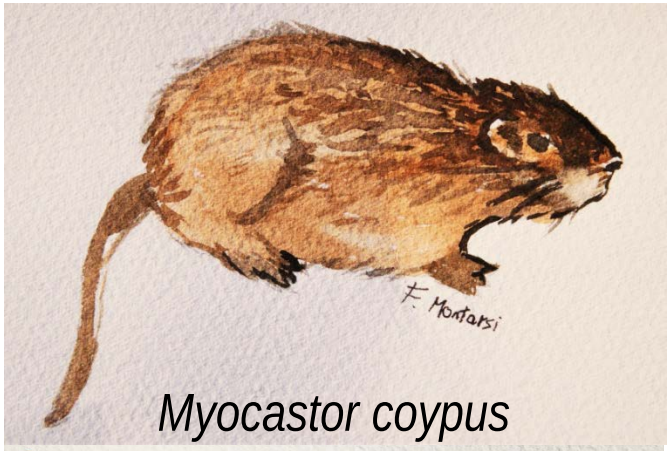
Latinne *et al.*, 2017. Genetic diversity and evolution of *Pneumocystis* fungi infecting wild Southeast Asian murid rodents. https://doi.org/10.1017/S0031182017001883


F. Montarsi

Pneumocystis screening in “alien” rodents in Italy

- ❑ Lungs collected and preserved at -20 degree
- ❑ Nested mtSSU and mtLSUrRNA PCR
- ❑ Real-time targeting a portion of the mtSSUrRNA
- ❑ ITS 1/2 – Inter-trascribed spacers of rRNA

Trapped and euthanized according to a project for the **control of allochthonous wildlife populations –**



Myocastor coypus

Callosciurus finlansonii

Danesi et al., 2017. Real-time PCR assay for screening *Pneumocystis* in free-living squirrels and river rats in Italy. Accepted in JVDI

Pneumocystis colonisation in wildlife

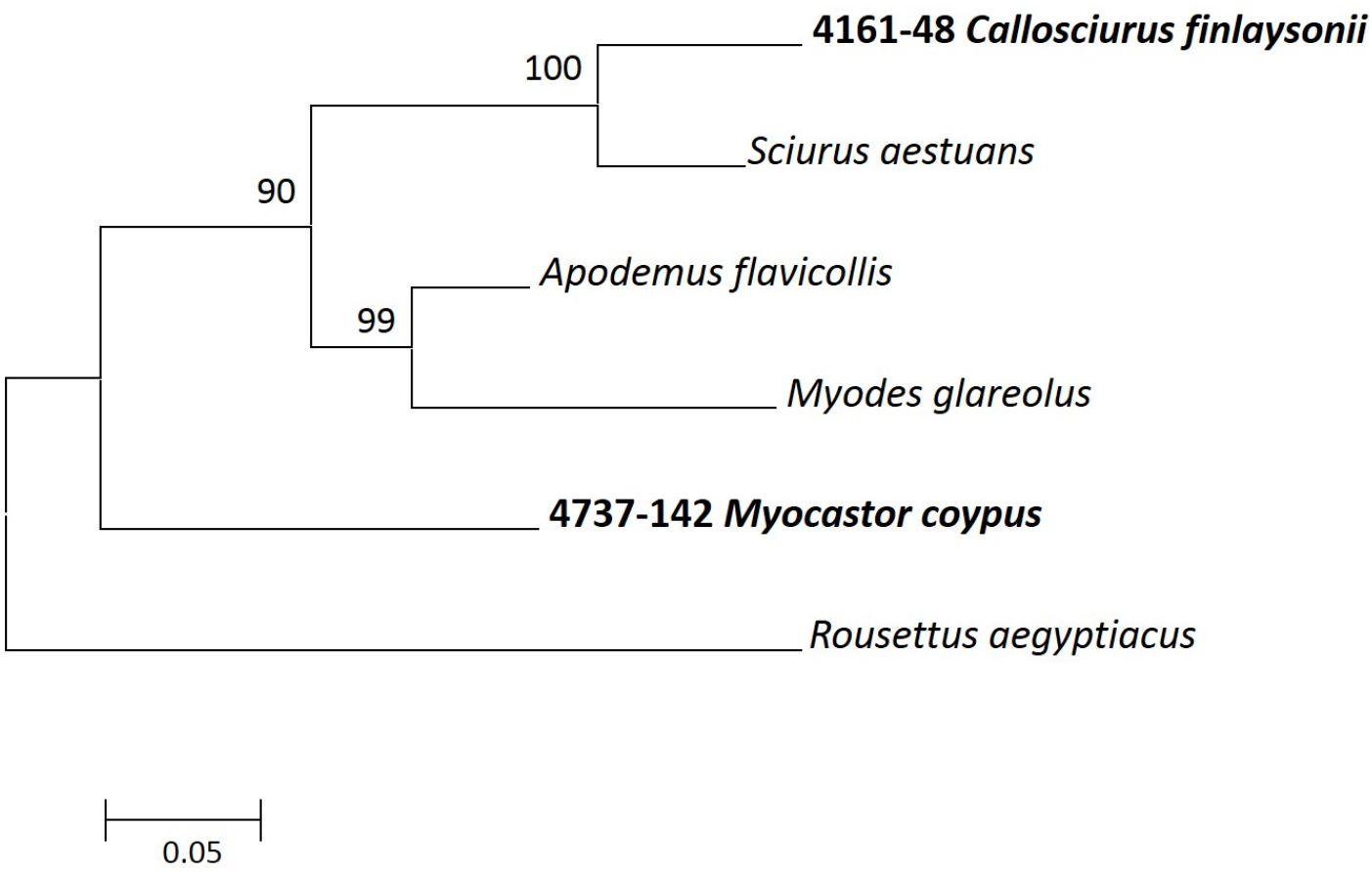
River rats (35/43; 81%)

Squirrels (20/85; 24%)

River rats were colonized more commonly than were squirrels

Danesi et al., 2017. Real-time PCR assay for screening *Pneumocystis* in free-living squirrels and river rats in Italy. Accepted in JVDI

Single Pneumocystis ST according host mammal



Concatenated Pneumocystis mtSSU and mtLSU rRNA sequence of Nested PCR

Danesi et al., 2017. Real-time PCR assay for screening *Pneumocystis* in free-living squirrels and river rats in Italy. Accepted in JVDI

Conclusions: lung co-infection

- 4/43 river rats co-infected with Emmonsia
- Among Pneumocystis positive animals, 2 squirrels co-infected with C. neoformans (Iatta et al., 2016)

Conclusions

- High prevalence (81%) - **airborne transmission** of Pneumocystis particles occurs when animals live in close contact with each other links transmission to specific behavioral traits or phases of an animal's life.

- River rats usually organize in **small matriarchal harems**

- **Vertical transmission**, as reported for rabbits and humans, has not been reported in rodents and might represent an efficient strategy for Pneumocystis to survive and propagate

Considerations

Primer set and PCR protocol work differently in different animal species

- ITS1 and 2 used previously in rodents (Danesi *et al*., 2016) worked with squirrels but coypus
- nested mtLSUrRNA PCR failed as well in both rodents - New primer sets were designed for rtPCR
- In our experience different volume of lung tissue investigated (% of the total lung volume) might influence the prevalence in animals suspected to be colonised with low prevalence Pneumocystis rates

Conclusions

- a “sequence-based” screening PCR – targeting a short amplicon of the mtSSUrRNA – increase the sensitivity in detecting Pneumocystis positive animals from lung tissue and BAL

- Because of the lack of culture, the phylogenetic species recognition (PSR) approach seems to be a promising and robust tool to describe new *Pneumocystis* spp