First report of *Rickettsia felis* in China

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**Abstract**

**Background:** *Rickettsia felis* is a recently described flea-borne spotted fever group *Rickettsia* that is an emerging human pathogen. Although there is information on the organism from around the world, there is no information on the organism in China.

**Methods:** We used a commercial ELISA to detect antibodies reactive against *R. felis* in blood samples and developed a PCR to detect the *gltA* of the organism in blood samples and external parasites.

**Results:** We found reactive antibodies in people (16%; 28/180), dogs (47%; 128/271) and cats (21%; 19/90) and positive PCRs with DNA from people (0.1%; 1/822), dogs (0.8%; 8/1,059), mice (10%; 1/10), ticks (*Rhipicephalus sanguineus*; 10%; 15/146), lice (*Linognathus setosus*; 16%; 6/37), fleas (*Ctenocephalides felis felis*; 95%; 57/60) and mosquitoes (*Anopheles sinensis*, *Culex pipiens pallens*; 6%; 25/428), but not from cats (0/135) or canine fecal swabs (0/43).

**Conclusions:** This is the first report of *R. felis* in China where there is serological and/or PCR evidence of the organism in previously reported [people, dogs, cats, ticks (*Rhipicephalus sanguineus*), fleas (*Ctenocephalides felis felis*) and mosquitoes (*Anopheles sinensis*, *Culex pipiens pallens*]) and novel species [mice and lice (*Linognathus setosus*)].

**Keywords:** *Rickettsia felis*, China, Serology, PCR

**Background**

Although tick-borne spotted fever group rickettsiae have been described in China [1], there is no information on the flea-borne emerging human pathogen *Rickettsia felis*. Described in 2001, *R. felis* appears to have the cat flea, *Ctenocephalides felis felis*, as its main vector and reservoir and can infect other arthropods (mosquitoes, ticks and mites) and mammals (rats, opossums, dogs, and cats). It is found worldwide and, in Asia, it has been definitively identified by molecular methods in fleas (Indonesia, Thailand, Afghanistan, South Korea, Laos, Malaysia, Taiwan), ticks (Japan), raccoons (Japan) and people (Taiwan, South Korea) [2,3]. To expand our knowledge on *R. felis* in Asia, we studied people, animals and arthropods from around China using serology and molecular techniques.

**Methods**

**Samples collection**

This study was approved by the Institutional Animal Care and Use Committee of Yangzhou University and the Institutional Review Board of Subei People's Hospital, China. Written permission was obtained from participants and owners of animals that participated in the study. People sampled in Jiangsu province (Figure 1) were apparently healthy individuals attending the Subei People's Hospital for routine health checks. Dogs sampled in Taixing of Jiangsu were apparently healthy animals in a breeding kennel while those from Gansu province were from a shelter. All other dog samples were obtained from patients with a variety of conditions attending local veterinary clinics. The cats sampled in Jiangsu were apparently healthy animals in a breeding kennel while those from Gansu province were from a shelter. All other dog samples were obtained from patients with a variety of conditions attending local veterinary clinics. The cats sampled in Jiangsu were apparently healthy animals in a breeding kennel while those from Gansu province were from a shelter. All other dog samples were obtained from patients with a variety of conditions attending local veterinary clinics. The cats sampled in Jiangsu were apparently healthy animals in a breeding kennel while those from Gansu province were from a shelter. All other dog samples were obtained from patients with a variety of conditions attending local veterinary clinics. The cats sampled in Jiangsu were apparently healthy animals in a breeding kennel while those from Gansu province were from a shelter. All other dog samples were obtained from patients with a variety of conditions attending local veterinary clinics.
captured in traps in Guangdong and the mosquitoes were captured with hand-nets in the environs of the Yangzhou University of Jiangsu.

Plasma and buffy coats from people, dogs, cats and wild mice (Figure 1) were stored at −80°C until DNA extraction. Rectal swabs from dogs and organs (spleen, liver and kidney) from the humanely euthanized wild mice were stored at −80°C in 800 μL of RNA/DNA Stabilization Reagent for Blood/Bone Marrow (Roche Molecular Biochemicals, Indianapolis) until DNA extraction. The external parasites collected from dogs and cats, and mosquitoes (Figure 1) were identified using standard morphological criteria and stored as above.

Serology assay
The *R. felis* EIA IgG Antibody Kit (Fuller Laboratory, USA) was used according to the manufacturer’s instructions with peroxidase-conjugated AffiniPure Goat Anti-Cat, Rabbit Anti-Dog, and Goat Anti-Mouse IgG (H + L) (Jackson ImmunoResearch Laboratories, USA) substituted as secondary antibodies for cat, dog and mouse/shrew assays, respectively. For human plasma, the cut-off level was determined following the manufacturer’s instructions that an index (OD value of test serum divided by the average OD values of the Cutoff Calibrator) above 1.2 should be considered positive. Plasma from cats, mice, shrews and dogs was regarded as positive.
if they gave an OD value above the mean plus three standard deviations of the respective negative control samples [4,5].

**DNA extraction**

Samples were thawed at room temperature and DNA was extracted from buffy coats, homogenized organs and arthropods [6], and canine rectal swabs with the QIAamp® DNA Blood Mini Kit (QIAGen, Valencia, USA), QIAamp® DNA Mini Kit, and QIAamp® DNA Stool Mini Kit, respectively, following the manufacturer’s protocol.

**PCR assays**

Using the Clustal Multiple Alignment Algorithm we identified a conserved region of the *gltA* in 20 representative *Rickettsia* species. Primers and probes were designed to amplify a 170-bp target using a FRET-PCR, and 446-bp and 353-bp targets using a nested-PCR (Figure 2). The PCRs were performed in a LightCycler® 480II PCR platform with hydroxymethylbilane synthase as an endogenous internal control [6]. Ten microliters of extracted DNA was tested in a 20 µL final volume of reaction mixture. Thermal cycling consisted of a denaturation step (2 min @ 95°C) and 18 high-stringency step-down cycles followed by

| A | upstream primer | fluorescein probe | LCRed-460 probe | downstream primer |
|---|-----------------|------------------|----------------|------------------|
| R. felis | TTRCAAATAGCAATAGAACTTGAAGCT | ACGGCCTTAAAGATGAATATTTTATTGAG | GAAAATTATATCCAAATGTTGATTTTTATTC | ACGGAACACCTAGCTGGAT |
| R. australis | | | | |
| R. akari | | | | |
| R. hanoi | | | | |
| R. parkeri | | | | |
| R. rickettsii | | | | |
| R. hoogstraali | | | | |
| R. japonica | | | | |
| R. prowazekii | | | | |
| R. typhi | | | | |
| R. typhi | | | | |
| R. africanus | | | | |
| R. sibirica | | | | |
| R. canadensis | | | | |
| R. conorii | | | | |
| R. helvetica | | | | |
| R. massiliae | | | | |
| R. zompoltimmas | | | | |
| R. montana | | | | |
| R. rhipicephali | | | | |
| R. slovaca | | | | |

**Figure 2** Alignment of the primers and probes for the *gltA*-based FRET-qPCR and nested PCR with 20 *Rickettsia* species. Panel A shows the nucleotide sequences of the primers and probes used in the FRET-qPCR and the corresponding sequences of 20 *Rickettsia* species. Panel B shows the nucleotide sequences of the primers used for the nested PCR. In both panels, dots indicate that nucleotides are identical to the primers. The nucleotides between oligonucleotides are not shown. The upstream primer and probes were used as shown while the downstream primer was used as an antisense oligonucleotide.
40 relaxed-stringency fluorescence acquisition cycles. The 18 high-stringency step-down thermal cycles were 6 × 1 sec @ 95°C, 12 sec @ 70°C, 8 sec @ 72°C; 9 × 1 sec @ 95°C, 12 sec @ 68°C, 8 sec @ 72°C; 3 × 1 sec @ 95°C, 12 sec @ 66°C, 8 sec @ 72°C. The relaxed-stringency fluorescence acquisition cycling consisted of 40 × 1 sec @ 95°C, followed by fluorescence acquisition of 8 sec @ 57°C, and 30 sec @ 72°C. Melting curve analysis for probes annealing to the PCR products was performed by monitoring the fluorescence from 38°C to 85°C with the first derivatives of F4/F1 being evaluated to determine the probe melting temperature ($T_m$). For nested-PCR, the PCR steps were the same as those in the FRET-PCR with the exclusion of the melting step. Positivity of samples was confirmed using gel electrophoresis with the SYBR® safe DNA Gel Stain (Invitrogen™, Carlsbad, CA, USA) and genomic sequencing conducted by a commercial company (GenScript, Nanjing, China). Two ultramer oligos (Integrated DNA Technologies, USA) containing portions of the gltA of *R. felis* and *R. typhi* were used to prepare quantitative standards (10⁴ to 10⁹ gltA copies/10 μL) and establish the sensitivity. All the PCR assays were performed with plasmid standards and sterile H2O as positive and negative controls, respectively.

### Results and discussion

The ELISAs showed high prevalences of antibodies to *R. felis* in people (16%; 28/180), dogs (47%; 128/271) and cats (21%; 19/90) (Figure 1, Table 1). Previous serosurveys have shown similar numbers of apparently healthy people in Colombia (18%), Spain (7%), Senegal (4%), and Kenya (3%) [7,8] are seropositive. There were no differences in age or complete blood count (CBC) parameters between sero-positive and sero-negative people. People that were ELISA positive had an average age of 45.03 years ±14.57 and their complete blood count parameters were: RBC: 4.76 × 10¹²/L ± 0.42, HCT: 43.52% ±3.57, WBC: 6.20 × 10⁹/L ±1.73, NE%: 58.61 ± 8.35, and PLT:

| Sample type | Source of samples | City | Coordinates | Sample number | Sero-positivity (%), pos/total samples | PCR-positivity (%), pos/total samples |
|-------------|-------------------|------|-------------|---------------|---------------------------------------|--------------------------------------|
| Human blood | Jiangsu           | Yangzhou | 32°N, 119°E | 822 | 15.6%, 28/180 | 0.1%, 1/822 |
| Dog blood   | Beijing           | Beijing  | 39°N, 116°E | 134 | 81.0%, 17/21 | 0.0%, 0/134 |
|             | Gansu             | Lanzhou  | 36°N, 103°E | 96 | 70.0%, 14/20 | 0.0%, 0/96 |
|             | Guangdong        | Guangzhou | 23°N, 113°E | 35 | 40.0%, 8/20 | 0.0%, 0/35 |
|             | Henan             | Zhengzhou | 34°N, 113°E | 102 | 60.0%, 12/20 | 0.0%, 0/102 |
|             | Inner Mongolia    | Huhhot   | 40°N, 111°E | 82 | 100.0%, 20/20 | 0.0%, 0/82 |
|             | Jiangsu           | Yangzhou | 32°N, 119°E | 50 | 25.0%, 5/20 | 0.0%, 0/50 |
|             |                   | Taizhou   | 32°N, 120°E | 111 | 0.0%, 0/10 | 0.0%, 0/111 |
|             |                   | Nanjing   | 32°N, 118°E | 61 | 10.0%, 1/10 | 0.0%, 0/61 |
|             |                   | Shanghai  | Shanghai   | 31°N, 121°E | 84 | 95.0%, 19/20 | 2.4%, 2/84 |
|             |                   | Shaanxi   | Yangling   | 34°N, 108°E | 56 | 75.0%, 15/20 | 0.0%, 0/56 |
|             |                   | Xinjiang  | Urumchi    | 43°N, 87°E | 86 | 40.0%, 8/20 | 0.0%, 0/86 |
|             |                   | Yunnan    | Kunming    | 25°N, 102°E | 162 | 12.9%, 9/70 | 5.8%, 6/162 |
| Cat blood   | Beijing           | Beijing  | 39°N, 116°E | 37 | 16.7%, 1/6 | 0.0%, 0/37 |
|             | Guangdong        | Guangzhou | 23°N, 113°E | 20 | 5.0%, 1/20 | 0.0%, 0/20 |
|             | Jiangsu           | Yangzhou | 32°N, 119°E | 38 | 29.7%, 11/37 | 0.0%, 0/38 |
|             | Shanghai          | Shanghai  | 31°N, 121°E | 50 | 22.2%, 6/27 | 0.0%, 0/50 |
| Lice        | Jiangsu           | Taizhou   | 32°N, 120°E | 37 | NA* | 16.2%, 6/37 |
| Tick        | Jiangsu           | Taizhou   | 32°N, 120°E | 146 | NA | 10.3%, 15/146 |
| Cat flea    | Jiangsu           | Yangzhou | 32°N, 119°E | 60 | NA | 95.0%, 57/60 |
| Mosquito    | Jiangsu           | Yangzhou | 32°N, 119°E | 664 | NA | 6.3%, 42/664 |
| Dog Rectal swab | Yunnan  | Kunming    | 25°N, 102°E | 43 | NA | 0.0%, 0/43 |
| Mouse blood | Guangdong        | Zhanjiang | 21°N, 110°E | 10 | NA | 0.0%, 0/10 |
| Liver       |                   |           |            | 10 | NA | 0.0%, 0/10 |
| Kidney      |                   |           |            | 10 | NA | 0.0%, 0/10 |
| Spleen      |                   |           |            | 10 | NA | 10.0%, 1/10 |
211.00 \times 10^9/L \pm 48.20. The average age of the seronegative people was 45.15 years \pm 14.61 and their blood parameters were RBC: 4.76 \times 10^{12}/L \pm 0.42, HCT: 43.55\% \pm 3.59, WBC: 6.20 \times 10^9/L \pm 1.74, NE%: 58.54 \pm 8.41, and PLT: 211.03 \times 10^9/L \pm 47.66.

Seropositive dogs were found in each area studied with prevalences from 13-100%, similar to the 51% reported in Spain and Australia, and \pm 13% in Brazil [9,10]. There were significantly fewer seropositive cats (5-30\% positive by PCR, despite many being seropositive were probably then not infected. Dogs, however, had negative serology and blood PCRs and our canine rectal swabs were negative by PCR. These up to 9\% of dogs are PCR positive [9]. Previously, PCR positive (0.8\%; 8/1,059), similar to Australia where [8]. Dogs from 2 of the 10 areas studied were also with no serological response as reported previously been an acute infection or an asymptomatic infection (0.1\%; 1/822), a twenty-seven year old man with a normal CBC who was seronegative. This might have on the biology of mosquitoes.

As found previously [13], all the cats we studied were negative by PCR, despite many being seropositive and many harboring PCR positive Ctenocephalides felis, cat fleas. Fleas were the only ectoparasites found on cats and almost all were PCR positive (95\%; 57/60), consistent with the very high levels of infection found worldwide and the generally accepted hypothesis that cat fleas are the primary arthropod vectors and reservoirs [3]. Our finding that dogs have a higher seroprevalence and are positive by PCRs supports the hypothesis that they, rather than cats, might be the main mammalian reservoir of R. felis [14].

The spleen of one Mus musculus was PCR positive for R. felis which is the first definitive report of the organism in mice. A PCR positive Rattus norvegicus has also been reported [15] and investigation into the role of rodents in the epidemiology of R. felis appears warranted.

All the amplicons we obtained in the above PCRs had identical sequences to those of the R. felis type strain URRWXCAl2 (CP000053). In addition the amplicons had identical sequences with other strains of R. felis including those with GenBank sequences JQ674484 (from Aedes albopictus mosquitoes, Libreville, Gabon) and JN375498 (from Canis familiaris in Southeastern Brazil). We submitted sequences obtained from two dogs to GenBank along with some of the sequences described below (Table 2).

Ten percent of the ticks (146 Rhipicephalus sanguineus) we collected from dogs were PCR positive. The sequences were all identical to R. felis (CP000053) except one (KJ440521) which was 99\% identical to R. felis (CP000053) and R. typhi (U59714). Sixteen percent (6/37) of the dog lice (all Linognathus setosus) were positive; four had amplicons identical to R. felis (CP000053) while two amplicons from dog lice (KJ440522) were similar to R. endosymbiotm (EU760765) (97\%), R. bellii (U59716) (96\%) and R. felis (CP000053) (86\%). While R. felis has been reported in R. sanguineus in South America [16], ours is the first report of the organism in lice. All the dogs with PCR positive lice or ticks were sero- and PCR negative for R. felis suggesting these arthropods might not be competent vectors.

Six percent (25/428) of the mosquitoes (32 Anopheles sinensis, 396 Culex pipiens pallens) were PCR positive with 23 (2 An. sinensis, 21 C. p. pallens) having sequences identical to R. felis (CP000053) and 2 (C. p. pallens) having 99\% and 96\% similarity. The latter was 99\% identical to a novel Rickettsia sp. (JN620082) found in An. gambiae in Africa which may be a new human pathogen [17]. Ours is the first report of R. felis and the new Rickettsia sp. in mosquitoes outside of Africa and the first of the organisms in An. sinensis and C. p. pallens. Further studies are indicated to determine the role of mosquitoes in the epidemiology of these rickettsias and the influence of the Rickettsia spp. might have on the biology of mosquitoes.

**Conclusions**

Our study indicates that R. felis occurs widely in China and infects a variety of previously reported (people, dogs,

| GenBank No. | Submission No. | Species |
|-------------|---------------|---------|
| KJ440515    | 1698905       | Dog - Canis lupus familiaris |
| KJ440516    | 1698976       | Dog - Canis lupus familiaris |
| KJ440517    | 1698981       | Louse - Linognathus setosus |
| KJ440518    | 1698985       | Louse - Linognathus setosus |
| KJ440519    | 1698989       | Mosquito - Anopheles sinensis |
| KJ440520    | 1699024       | Mosquito - Culex pipiens pallens |
| KJ440521    | 1699027       | Tick - Rhipicephalus sanguineus |
| KJ440522    | 1699029       | Louse - Linognathus setosus |
| KJ440523    | 1699030       | Mosquito - Anopheles sinensis |
cats, ticks, fleas and mosquitoes) and novel species (mice and lice). Further studies are indicated to investigate the epidemiology and transmission mechanisms of R. felis, particularly in mosquitoes, lice and mice.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
CW, JZ, and PK designed the experiment. JZ, GL, ZZ, DY, LW and SGL performed the experiment. CW and PK wrote the manuscript. All authors read and approved the final version of manuscript.

Acknowledgements
This project was supported by grant from the National Natural Science Foundation of China (NO: 31472225) and the Priority Academic Program Development of Jiangsu Higher Education Institutions, Yangzhou, Jiangsu, P. R. China.

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Received: 30 June 2014 Accepted: 3 December 2014
Published online: 16 December 2014

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