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Isolation and Characterization of *Rickettsia finnyi*, Novel Pathogenic Spotted Fever Group *Rickettsia* in Dogs, United States

Appendix

Rickettsia finnyi sp. nov strain 2024-CO-Wats M61 glycyl aminopeptidase qPCR

The *R. finnyi* sp-sp qPCR primers (5′CTTAGAAATGGGGACATA GTTAATG 3′ and 5′GTATATATCTTTTTTATTAGCACTTG 3′) and probe (6-FAM/ZEN- ATCAGATAG TTACAATCAACACAATTAAAGGC-IBFQ) were created (Integrated DNA Technologies, http://www.idtdna.com) to amplify a 147-bp region of the M61 glycyl aminopeptidase gene based on sequence alignment (Geneious Prime version 2021.1.1, https://www.geneious.com) with *Rickettsia* sp. 2019-CO-FNY M61 glycyl aminopeptidase gene region (OQ383420) and multiple *Rickettsia* spp. sequences available in NCBI GenBank (Appendix Figure 1). Amplification assays were performed in CFX96 Real-Time Detection System combined with C1000 Thermal Cycler (Bio-Rad, USA). *Rickettsia finnyi* sp-sp qPCRs contained 12.5 µL Primetime Gene Expression Master Mix (Integrated DNA Technologies, http://www.idtdna.com), primers at 0.6 µM and probe at 0.4 µM, 5 µL of DNA template and molecular-grade water to a final volume of 25 µL. Thermocycler conditions consisted of an initial denaturation step at 98°C for 3 minutes, followed by 40 cycles of 98°C for 15 s and 60°C for 30 s.

Major facilitator superfamily (MFS) transporter qPCR

Primers (5′ CAAGCAGTCGGATTACTTTC 3′ and 5′ AAACAACTACAATCTTACGTCC 3′) were designed to amplify a major facilitator

superfamily (MFS) transporter gene region for retrospective assessment of a mutation acquired in culture. Amplification assays were performed in CFX96 Real-Time Detection System combined with C1000 Thermal Cycler (Bio-Rad, USA). *Rickettsia finnyi* sp-sp qPCRs contained 12.5 µL Primetime Gene Expression Master Mix (Integrated DNA Technologies, http://www.idtdna.com), primers at 0.4 µM, 5 µL of DNA template and molecular-grade water to a final volume of 25 µL. Thermocycler conditions consisted of an initial denaturation step at 98°C for 3 minutes, followed by 40 cycles of 98°C for 15 s, 58°C for 15 s and 72°C for 15 s.

All PCR assays were run with negative molecular-grade water, a negative control of known uninfected canine DNA, and a positive control.

Rickettsia sp. 2024-CO-Wats Cultures.

Cell cultures 030D, DH82 and Vero E3 grown in varying culture containers were infected with *Rickettsia* sp. 2024-CO-Wats and monitored through culture supernatants or cell suspensions collected at various passages (P) (Appendix Tabel 2). DNA was extracted from 100 µL using a DNA extraction kit (Qiagen, https://www.qiagen.com) and monitored by *Rickettsia* 23s-5s ITS and GAPDH qPCRs. Fold changes in *Rickettsia* were calculated as $2^{-\Delta\Delta Cq}$, in which $\Delta\Delta Cq = (Cq_{Rick\ 23–5} - Cq_{GAPDH})$ timex – $(Cq_{Rick\ 23–5} - Cq_{GAPDH})$ time0 (Appendix Figure 2). At P4, one *Rickettsia* sp. 2024-CO-Wats culture from each cell line replicate was selected for continued maintenance, where 030D-P24 and DH82-P22 were maintained for 156 days and VE6-P4 cells for 108 days

Immunofluorescence staining

Infected cells were seeded on Nunc Lab-Tek II CC2 8-well chamber slides (ThermoFisher, https://www.thermofisher.com) from the following cultures and passages: 030D-P9, DH82-P8, and VE6-P2. At ~90% confluency, cells were rinsed with PBS and treated with 100 µg/ml of gentamicin sulfate for 2.5 h. Cells were washed 2 times to remove extracellular bacteria and residual antibiotics. They were fixed with 10% neutral buffered formalin for 10 min, permeabilized with 0.3% Triton X-100 in PBS at room temperature for 30 min and blocked with goat serum, Tween-20, and 0.5% of powdered nonfat milk for 1 h. Cells were incubated overnight at 4°C with canine serum diluted at 1:500 in a humidified chamber. Serum samples

used for incubation were derived from dogs numbered 2 and 3 infected with *Rickettsia* sp. 2024-CO-Wats that were cross-reactive against *R. rickettsii* IFA at 1:8192 (Table 1). The negative serum control was obtained from an archived, canine sample previously tested by a comprehensive tick-borne disease panel that was PCR negative and serologically nonreactive for all tick-borne pathogens tested, including *R. rickettsii* IFA. Infected cells were incubated with a fluorescein Isothiocyanate (FITC)-conjugated goat anti-dog IgG (H+L) antibody (Sigma-Aldrich, https://www.sigmaaldrich.com) for 1 h at room temperature. A secondary antibody control (blocking solution instead of canine serum) was performed. Nuclei were counterstained using DAPI (ThermoFisher, https://www.thermofisher.com). Between sequential steps (except after the blocking step), cells were washed 3 times with PBS. Coverslips were mounted with Prolong Gold antifade reagent (Invitrogen, https://www.thermofisher.com). Images were acquired with BZ-X810 Keyence.

Appendix Table 1. A summary of conditions used for *Rickettsia* sp. 2024-CO-Wats inoculation in Vero E6 (VE6), 030D, and DH82 cell lines and which conditions produced growth.

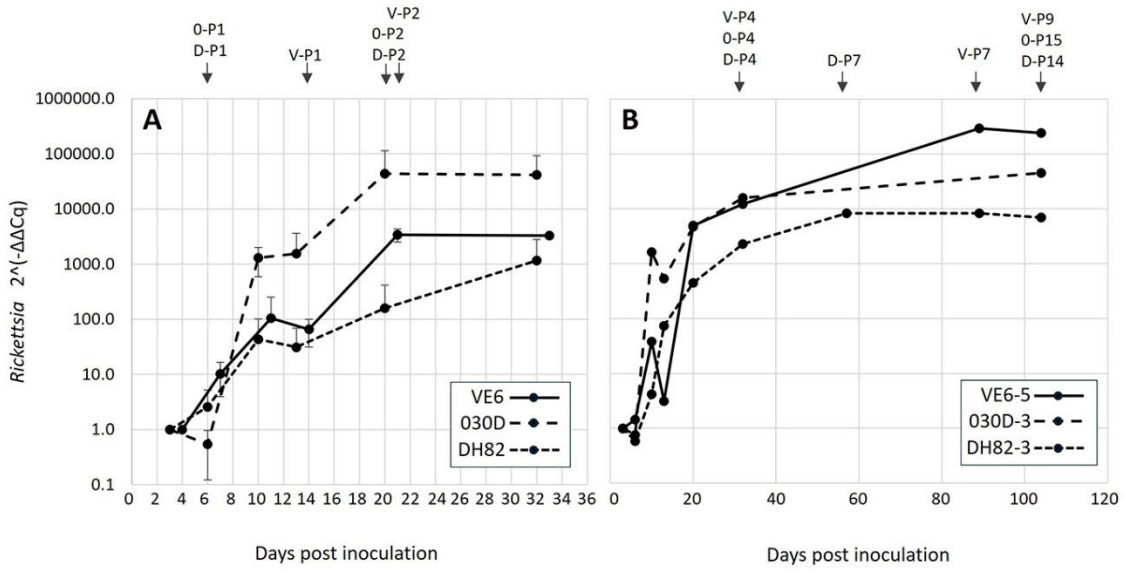
Culture*	Container surface area (cm^2)	Cell confluency (%)	Blood volume (µL)†	Days between blood collection to inoculation	Media	Media volume (mL)	Incubation (days)	Growth detected‡
VE6–1	7mL Tube (5.5)	95	100	7	DMEM 5% FBS	1.5	12	Yes
VE6–2	7mL Tube (5.5)	95	100	7	DMEM 5% FBS	1.5	12	Yes
VE6–3	7mL Tube (5.5)	50	100	7	DMEM 5% FBS	1.5	12	Yes
VE6–4	7mL Tube (5.5)	50	100	7	DMEM 5% FBS	1.5	12	Yes
VE6–5	T25 Flask (25)	75	300	8	DMEM 5% FBS	4.5	11	Yes
030D-1	6-well Plate (9.5)	75	100	8	RPMI 10% FBS	1.5	4	Yes
030D-2	6-well Plate (9.5)	75	100	8	RPMI 10% FBS	1.5	4	Yes
030D-3	6-well Plate (9.5)	75	100	8	RPMI 10% FBS	1.5	4	Yes
DH82–1	6-well Plate (9.5)	75	100	8	RPMI 10% FBS	1.5	4	No
DH82–2	6-well Plate (9.5)	75	100	8	RPMI 10% FBS	1.5	4	No
DH82–3	6-well Plate (9.5)	75	100	8	RPMI 10% FBS	1.5	4	Yes

*Numbers after the cell lines represent replicates. DMEM, Dulbecco's minimum essential medium; FBS, fetal bovine serum; RPMI, Roswell Park Memorial Institute-1640 Medium, GlutaMAX supplement; SPG, sucrose-phosphate-glutamate buffer.
†Combined volume of EDTA whole blood and SPG at a 1:1 ratio.
‡Growth was initially determined based on *Rickettsia* 23S-5S ITS and *R. finnyi*-specific M61 qPCR having lower Cq values than the diagnostic blood sample.

Appendix Table 2. *Rickettsia* spp. used in genomic comparison with 2024-CO-Wats using OrthoANI and dDDH GGDC.

Rickettsia genome compared with R. finnyi CP170741.1	OrthoANI value (%)	dDDH GGDC f1	dDDH GGDC f2	dDDH GGDC f3	Diff. G+C (%)
R. conorii raoultii CP098324.1	96.86	91.9	70.6	91.0	0.19
R. montanensis CP003340.1	96.81	94.0	69.7	92.5	0.26
R. slovaca CP002428.1	96.78	92.3	70.5	91.3	0.19
R. peacockii CP001227.1	96.75	87.1	69.5	86.8	0.30
R. honei AJTT01	96.74	92.3	69.1	91.0	0.11
R. japonica AP017600.1	96.68	91.6	69.5	90.5	0.01
R. parkeri CP040325.1	96.66	93.0	68.6	91.5	0.14
R. sibirica AABW01	96.63	91.4	68.5	90.1	0.16
R. conorii AE006914.1	96.60	91.9	68.7	90.5	0.13
R. massiliae CP003319.1	96.55	89.9	68.0	88.8	0.29
R. philipii CP003308.1	96.55	93.8	67.5	91.9	0.16
R. rhipicephali CP003342.1	96.54	90.0	68.3	88.9	0.09
R. parkeri CP069388.1	96.52	91.2	67.5	89.8	0.15
R. parkeri CP003341.1	96.52	91.9	67.8	90.5	0.13
R. parkeri LAOO01000001.1	96.51	91.9	67.9	90.5	0.13
R. africae CP001612.1	96.51	91.6	67.5	90.1	0.10
R. rickettsii str. 'Sheila Smith' CP121767.1	96.48	92.6	66.9	90.8	0.16
R. rickettsii str. AZ-5 CP098688.1	96.44	92.0	66.8	90.3	0.15
R. conorii heilongjiangensis JAXOFY01	96.43	93.4	67.4	91.6	0.03
R. rickettsii str. Morgan CP006010.1	96.39	92.0	66.7	90.3	0.15
R. rickettsii str. Iowa CP000766.3	96.39	92.1	66.7	90.3	0.14
R. amblyommatis CP012420.1	96.28	87.0	65.6	85.9	0.10
R. tamurae buchneri JFKF01	94.24	55.9	54.4	56.4	0.17
R. monacensis LN794217.1	94.16	64.3	53.5	63.5	0.08
R. tamurae CCMG00000000.1	93.57	69.4	52.1	67.5	0.16
R. hoogstraalii CCXM01	92.77	65.4	47.5	62.7	0.07
R. helvetica CM001467.1	92.72	64.7	48.4	62.4	0.09
R. asembonensis JWSW01	92.65	62.3	47.9	60.3	0.07
R. tillamookensis CP060138.2	92.28	64.2	46.1	61.3	0.09
R. felis JSEL01	92.11	57.2	46.3	55.5	0.13
R. australis CP003338.1	91.77	65.6	44.1	61.7	0.02
R. akari CP000847.1	91.31	64.0	42.0	59.7	0.03
R. canadensis CP003304.1	88.62	51.3	36.4	47.6	1.29
R. oklahomensis CP157197.1	88.48	52.4	35.7	48.2	1.60
R. prowazekii CP003391.1	87.42	43.9	33.1	40.7	3.30
R. typhi CP003397.1	87.38	41.8	32.4	38.8	3.39
R. bellii CP015010.1	81.66	19.4	27.4	19.4	0.69

Appendix Figure 1. A sequence alignment depicting the oligonucleotides for the *Rickettsia* M61 glycyl aminopeptidase qPCR aligned with *Rickettsia finny* sp. nov. and multiple *Rickettsia* spp. sequences available in NCBI GenBank.



Appendix Figure 2. Line graphs depicting the relative growth curve of *Rickettsia finny* sp. nov. 2024-CO-Wats in replicates of Vero E6 (VE6), 030D and DH82 cells measured to 33 days (A) and individual cultures measured to 104 days (B) after inoculation with a naturally infected dog blood sample. *Rickettsia* growth is represented by *Rickettsia* 23s-5S ITs qPCR Cq values normalized to host GAPDH Cq values and fold change (2^(-ΔΔCq) relative to first day of testing after inoculation. Passages are indicated with arrows. 0-P, 030D passages; D-P, DH82 passages; V-P, VE6 passages.