Review

Tick-Borne Colpodella Species Infections: Time for a New Integrated Approach to Understand Transmission and Pathogenicity

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Review

Tick-Borne *Colpodella* Species Infections: Time for a New Integrated Approach to Understand Transmission and Pathogenicity

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Simple Summary: *Colpodella* species are free-living protists that are increasingly detected in ticks infesting animals and may represent an emerging zoonosis. We reviewed reports of *Colpodella* spp. detection in ticks and infection in human and animal hosts reported in the literature. Since 2012, twenty cases of *Colpodella* spp. detection in ticks, host blood and fecal samples have been reported. In 2024 alone, 5 cases were reported and in 2025, 1 new case has been reported. The same *Colpodella* spp. and strains identified in different tick species by polymerase chain reaction (PCR) using primers targeting the 18S rRNA gene was also identified in human blood and cerebrospinal fluid (CSF), suggestive of zoonotic infections. Tick bites in various animals and in a human infection have been reported. *Colpodella* spp. have also been reported in two human cases of relapsing fever and in cases of diarrhea in animals. Investigations into the pathogenicity of *Colpodella* spp. need to include morphological descriptions of *Colpodella* species identified in ticks, human and animal specimens. A clear understanding of life cycle stages of *Colpodella* spp. will aid accurate diagnosis of *Colpodella* spp. infections.

Abstract: *Colpodella* species are free-living predatory protists that prey on algae, ciliates and bodonids using myzocytosis. *Colpodella* species have been reported in human and animal infections. Polymerase chain reaction (PCR) using primers targeting 18S rRNA genes of *Cryptosporidium* and piroplasms has identified *Colpodella* species in arthropods, host blood, and feces demonstrating the phylogenetic closeness of *Colpodella* species to the apicomplexa. However, in both human and animal infections, life cycle stages of *Colpodella* present in arthropods and infected hosts are unknown. In this review we provide an overview of widespread occurrence of *Colpodella* species in ticks, and pathogenicity in humans and animals. We discuss methods for culture and microscopy that can aid diagnosis. Phylogenetic tree analysis of *Colpodella* species identified using 18S rRNA demonstrates that the *Colpodella* species identified in different geographic regions represent different species and strains that may impact virulence and zoonotic transmission. There is a pressing need to culture *Colpodella* species, and to stain cells for morphological identification. This will aid molecular investigations aimed at identifying molecular markers of *Colpodella* spp. facilitating transmission, survival and pathogenesis in hosts, and determining which species and strains to prioritize for the risk of zoonotic infections to humans and for infections in animals.

Keywords: Apicomplexa; *Babesia* species; *Colpodella* species; *Colpodella* life cycle; *Cryptosporidium* spp.; Myzocytosis; Piroplasms; tickborne infections; zoonosis

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1. Introduction

Colpodella species are free-living protist predators that are found in a variety of environmental locations. *Colpodella* spp. are phylogenetically related to the pathogenic apicomplexa like *Plasmodium* spp. and *Babesia* spp. [1]. Several *Colpodella* species are recognized based on ultrastructure and the presence of an apical complex, such as *Colpodella angusta, C. edax*, *C. pseudoedax*, *C. pugnax*, *C. vorax*, *C. unguis*, *C. tetrahymenae*, *C. gonderi* and *C. perforans* [2, 3, 4, 5, 6]. *Colpodella turpis* has been identified using differential interference contrast microscopy (DIC) but has not been characterized by ultrastructure [2]. *Colpodella pontica* is now known as *Voromonas pontica* due to phylogenetic and morphological characterizations that led to a change in the genus name [6]. Additional efforts aimed at redefining taxonomic nomenclature and positions have been initiated for some of the other *Colpodella* species [6]. However, additional life cycle characteristics, including morphological, molecular, cell biology, genetic and biochemical data will be needed to correctly assign the new nomenclature to *Colpodella* spp. The life cycle of *Colpodella* species consists of trophozoite and cyst stages [2, 3, 7, 8]. *Colpodella* trophozoites are fusiform ("banana shaped") in shape with a curved rostrum at the anterior end and have a length of between 5 μm-22 μm depending on the species [2]. For example, *Colpodella gonderi* is approximately 5 μm, *C. vorax* is 8-12 μm, *C. pugnax* is 7-15 μm, *C. turpis* is 14-22 μm and *C. edax* is 10-18 μm [2, 4]. The width of the trophozoites varies in size from 2 μm-10 μm. *Colpodella* sp. ATCC 50594 maintained in Hay medium culture containing its prey *Parabodo caudatus*, was shown to have juvenile trophozoites that emerge from cysts, and feed by myzocytosis on *P. caudatus* [7, 8]. The trophozites are 7-9 μm long and the posterior food vacuole formed after feeding is 2.5 μm to 6.4 μm in diameter. The round to oval cyst stages of *Colpodella* spp. are 5-6.7 μm in diameter [4]. After the trophozoites differentiate into pre-cysts still containing large posterior food vacuoles, the cyst divides to release juvenile trophozoites that repeat the cycle [7, 8]. Cell division results in the production of two or more trophozoites. In *Colpodella* sp. ATCC 50594 asymmetric cell division takes place and asynchronous development of the trophozoites occurs within the cyst [7]. Some *Colpodella* species such as *C. pseudoedax* do not encyst and divide by longitudnal fission. Similarly, oblique-transversal cell fission was identified in *C. unguis* which also possesses trichocysts [4, 5]. Two types of cysts are formed, transient and resting cysts. The former excysts during the most active stage of the culture resulting in the release of juvenile trophozoites, while the latter can persist in culture for up to fourteen days and then they are used for subculture to seed new cultures [7, 8].

1.1. Geographic distribution of Colpodella species

Once thought to be rare in the environment, *Colpodella* species have been detected globally in arthropods [9, 10, 11, 12, 13, 14, 15], soil, grass, among dead leaves, cattle manure, fecal samples, on damp wood chips and surface beach sand [16, 17, 18, 19], in dairy cattle manure [20], thrombolitic microbialites [21], wastewater, freshwater, ponds and marine environments [16] and in blood, cerebrospinal fluid (CSF), urine and skin [22, 23, 24, 25, 26, 27] (Table 1). The global range of *Colpodella* species is cosmopolitan and includes countries in North, South, East and West Africa, North, Central and South America, Asia, Europe, Greenland in the Arctic [28], Antarctica [29] and in Australia [30], and New Zealand (Table 1). *Colpodella* species have been identified in six genera of ticks and in a biting fly found on several types of animals including horses, cattle, goats, sheep, dogs, cats, pangolins, fox, duck, Eurasian Coot, in cultures of mosquito larvae and in association with sea bass [19, 31] (Table 1). *Colpodella* species have been reported in four human infections consisting of two cases of relapsing fever, a tick-borne infection and a case of urinary tract infection [22, 23, 26, NCBI accession number MF594625]. *Colpodella* spp. infections may be zoonotic since the same *Colpodella* species and strains have been identified in both human, animals and ticks [11, 12, 14, 19, 22, 23]. Roughly 75 % of human infectious and parasitic diseases are zoonotic [32] (https://www.who.int/news-room/fact-sheets/detail/zoonoses accessed 1/11/25). In order to develop preventive measures and appropriate treatments, the status of *Colpodella* spp. as opportunistic or

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zoonotic parasites will need to be established. Molecular markers including virulence factors of *Colpodella* species are unknown, host specificity is unknown and the significance of co-infections with bacteria, piroplasms and *Cryptosporidium* spp. in hosts are also unknown. We recently identified Kelch 13 and coronin genes in *Colpodella* sp. ATCC 50594 as markers for endocytosis [33]. However, additional markers will need to be identified to aid characterization of stages involved in transmitting infection and contributing to pathogenesis. *Colpodella* spp. prey on bodonids, ciliates and algae as free-living protists, using the process of myzocytosis to aspirate nutrients from their prey. Cytoplasmic contents of the prey or whole prey can be aspirated into a posterior food vacuole of the predator [2, 3, 4, 5, 6, 7]. The ectoparasitic *Colpodella*, consisting of *C. tetrahymenae* and *C. gonderi* prey on ciliates and attach for periods of prolonged feeding on their prey [6, 34]. Trophozoites of the model *Colpodella* sp. ATCC 50594 carry out endocytosis, in addition to myzocytosis demonstrating that nutrient uptake can occur through predation and endocytosis [35]. The mode of *Copldella* spp. survival in arthropod and vertebrate hosts is unknown. During myzocytosis, *Colpodella* spp. can aspirate cytoplasmic contents along with organelles or whole prey into a posterior food vacuole suggesting that attachment to host cells may occur and that uptake of whole cells or destruction and aspiration of host cell contents may occur. *Colpodella* spp. can also feed intermittently on different prey by moving from one prey to another aspirating cytoplasmic contents [2, 3, 35]. The identification of protists serving as prey for *Colpodella* species has not been described in the arthropod and vertebrate hosts reported. Whether *Colpodella* spp. feeds by myzocytosis, trogocytosis or phagotrophy within hosts is unknown. *Colpodella* spp. have been identified in ticks co-infected with the piroplasms *Babesia* spp. and *Theileria* spp. and detected in fecal samples from diarrheic small ruminants, ostrich and calves that were also infected with *Cryptosporidium* spp. (Table 1). *Colpodella* spp. were also detected in the feces of a goat, fox, duck and Eurasian Coot [18].

Table 1. Global distribution of *Colpodella* species in arthropods, vertebrates and environmental sources, retrieved from NCBI (https://www.ncbi.nlm.nih.gov/nuccore) and from published studies [16].

	Organism Name	Source	Animal	Tick/Biting flies	Country
1.	*Colpodella* sp. QYi-2023b	Ticks infesting goat and dog	Goat	*Haemaphysalis longicornis*	China: Yiyuan county Shangdong
2.	*Colpodella* sp. QYi-2023a	Ticks infesting goat	Goat	*Haemaphysalis longicornis*	China: Yi yuan county Shangdong
3.	*Colpodella* sp. isolate 103	Blood	Horse	N/A	China
4.	*Colpodella sp.* isolate 115	Blood	Horse	N/A	China
5.	*Colpodella* sp. RRJ-2016 isolate T18	N/A	N/A	N/A	China
6.	*Colpodella* sp. RRJ-2016 isolate T17	N/A	N/A	N/A	China
7.	*Colpodella* sp. HLJ	Woman with neurological symptoms	N/A	Tick	China
8.	*Colpodella* sp. HLJ	ticks infesting dog	Dog	*Haemaphysalis longicornis*	China: Yiyuan County, Shandong
9.	Uncultured *Colpodella*	Blood	N/A	N/A	(China)
10.	*Colpodella* sp.	Blood	Dog	N/A	China: Guiyang
11.	*Colpodella* sp.	Blood	Cat	N/A	China: Guiyang
12.	*Colpodella* sp.	N/A	N/A	N/A	China

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13. *Colpodella* sp.	N/A	*Panthera tigris altaica* (Amur tiger)	N/A	China
14. *Colpodella* sp.	N/A	*Panthera tigris altaica* (Amur tiger)	N/A	China
15. *Colpodella* sp.	N/A	*Panthera tigris altaica* (Amur tiger)	N/A	China
16. *Colpodella* sp.	N/A	*Panthera tigris altaica* (Amur tiger)	N/A	China
17. *Colpodella tetrahymenae*	N/A	*Panthera tigris altaica* (Amur tiger)	N/A	China
18. *Colpodella* sp.	N/A	N/A	*Rhipicephalus microplus*	China
19. *Colpodella* sp.	N/A	N/A	*Rhipicephalus microplus*	China
20. *Colpodella tetrahymenae*	Wastewater	N/A	*Dermacentor everestianus*	China: Qinghai
21. *Colpodella* sp.	N/A	N/A	*Dermacentor nuttalli*	china: Qinghai
22. *Colpodella* sp.	N/A	N/A	*Dermacentor nuttalli*	china: Qinghai
23. *Colpodella* sp.	N/A	N/A	*Dermacentor nuttalli*	China: Qinghai
24. *Colpodella tetrahymenae*	N/A	N/A	*Dermacentor everestianus*	China: Qinghai
25. *Colpodellidae* sp.	man with relapsing fever	*Homo sapiens*	N/A	China
26. *Colpodellidae* sp.	woman with relapsing Babesia-like illness	*Homo sapiens*	N/A	China
27. *Colpodella sp.* clone pangolin 18_Tick2	*Amblyomma javanense*	Pangolin	Tick	China: Guangzhou
28. *Colpodella sp.* clone pangolin 17_Tick1	*Amblyomma javanense*	Pangolin	Tick	China: Guangzhou
29. *Colpodella sp.* clone pangolin 16_Tick2	*Amblyomma javanense*	Pangolin	Tick	China: Guangzhou
30. *Colpodella sp.* clone pangolin 12_Tick1	*Amblyomma javanense*	Pangolin	Tick	China: Guangzhou
31. *Colpodella sp.* clone pangolin 11_Tick2	*Amblyomma javanense*	Pangolin	Tick	China: Guangzhou
32. *Colpodella sp.* clone pangolin 4_Tick1	*Amblyomma javanense*	Pangolin	Tick	China: Guangzhou
33. *Colpodella* sp.	N/A	N/A	Tick	Pakistan
34. *Colpodella* sp.	N/A	N/A	Tick	Pakistan
35. *Colpodella* sp.	N/A	N/A	Tick	Pakistan
36. *Colpodella* sp.	N/A	N/A	Tick	Pakistan

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37. *Colpodella* sp.	N/A	N/A	Tick	Pakistan
38. *Colpodella* sp.	N/A	N/A	Tick	Pakistan
39. *Colpodella* sp.	tick P03 collected from cattle	Cattle	*Rhipicephalus* (*Boophilus*) *microplus*; sex: female	Russia
40. *Colpodella angusta*	N/A	N/A	N/A	Russia
41. *Colpodella* sp.	N/A		*Stomoxys indicus*	Thailand: Nakhon Si Thammarat
42. *Colpodella* sp.	Blood	Dog		Cambodia
43. Uncultured *Colpodella*	N/A	*Bos taurus*	N/A	Japan
44. *Colpodella* sp.	Feces	Fox (*Vulpes vulpes indutus*)	N/A	Nicosia, Cyprus
45. *Colpodella* sp.	Feces	Duck *Anas* spp.	N/A	Nicosia, Cyprus
46. *Colpodella* sp.	Feces	Duck *Anas* spp.	N/A	Nicosia, Cyprus
47. *Colpodella* sp.	Feces	Eurasian Coot (*Fulica atra*)		
48. *Colpodella* sp.	Feces	Goat (*Capra hircus*)	N/A	Nicosia, Cyprus
Colpodella tetrahymenae 49.	N/A	N/A	N/A	Portugal
Colpodella angusta	Identified with *Amyloodinium ocellatum* (dinoflagellate ectoparasite)	Sea bass *Dicentrarchus labrax*	N/A	Portugal
50. Uncultured *Colpodella* clone PL31	Skin, dried ear fragments, Warta Mouth National Park, western Poland	*Procyon lotor*	N/A	Poland
51. Uncultured *Colpodella* clone PL31	Skin, dried ear fragments, Warta Mouth National Park, western Poland	*Procyon lotor*	N/A	Poland
52. Uncultured *Colpodella*	slow sand filter column for wastewater treatment, Leipzig	N/A	N/A	Germany
53. *Colpodella angusta*	Marine sample		N/A	Germany Helgoland
54. Uncultured *Colpodella*	lake water filtered through 3 um from lake Esch sur Sure, depth 0m	N/A	N/A	Luxembourg
55. *Colpodella* sp.	Ticks	Cattle and goats	*Rhipicephalus bursa*	Italy
56. *Colpodella* sp.		Lake water		France
57. *Colpodella angusta*	Feces of calves with diarrhea		N/A	Turkey: Nevsehir
58. *Colpodella gonderi*	Woman with urinary tract infection		N/A	Romania

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	(identified with *Colpoda steinii*)			
59. *Colpodella* sp. TT-2023	whole body of *Hyalomma dromedarii*	Camel	*Hyalomma dromedarii*	Egypt:Luxor
60. *Colpodella* sp. TT-2023	whole body of *Hyalomma dromedarii*	Camel	*Hyalomma dromedarii*	Egypt:Aswan
61. *Colpodella* sp. TT-2023	whole body of *Hyalomma dromedarii*	Camel	*Hyalomma dromedarii*	Egypt:Luxor
62. *Colpodella* sp. TT-2023	whole body of *Hyalomma dromedarii*	Camel	*Hyalomma dromedarii*	Egypt:Aswan
63. Uncultured *Colpodella*	Feces	Sheep	N/A	Nigeria
64. Uncultured *Colpodella*	Feces	Sheep	N/A	Nigeria
65. Uncultured *Colpodella*	Feces	Sheep	N/A	Nigeria
66. Uncultured *Colpodella*	Feces	Sheep	N/A	Nigeria
67. Uncultured *Colpodella*	Feces	Sheep	N/A	Nigeria
68. Uncultured *Colpodella*	Feces	Sheep	N/A	Nigeria
69. *Colpodella* sp.	tick P03 collected from cattle	Cattle	*Rhipicephalus* (*Boophilus*) *microplus*; sex: female	Mozambique
70. *Colpodella* sp.	Blood	Cattle and wildlife		Zambia
71. *Colpodella* sp.	N/A	Bovine	N/A	Brazil
72. *Colpodella* sp.	N/A	Bovine	N/A	Brazil
73. *Colpodella* sp.	N/A	Bovine	N/A	Brazil
74. *Colpodella* sp.	N/A	Bovine	N/A	Brazil
75. *Colpodella* sp.	N/A	Bovine	N/A	Brazil
76. *Colpodella* sp.	N/A	Bovine	N/A	Brazil
77. Uncultured *Colpodella*	tropical floodplain lake	N/A	N/A	Brazil
78. *Colpodella tetrahymenae*	N/A	N/A	N/A	Costa Rica
79. *Colpodella angusta*	laboratory culture	N/A	N/A	Canada: Vancouver
80. *Colpodella angusta*	laboratory culture	N/A	N/A	Canada: Vancouver

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81. *Clopodella angusta*	Damp wood chip and surface sand, Locarno beach	N/A	N/A	Canada Vancouver
82. *Colpodella angusta*	Wood chip on the beach	N/A	N/A	Canada Boundary Bay
83. *Colpodella angusta.*	Soil from UBC endowment lands	N/A	N/A	Canada Vancouver
84. Uncultured *Colpodella*	cave and mine	N/A	N/A	New York, USA
85. *Colpodella* sp. ATCC 50594	Brown woodland soil, Gambrill Park	N/A	N/A	Maryland, USA
86. *Colpodella angusta* FL1	Fresh water laboratory dishes with mosquito larvae, Rutgers University	Mosquito larvae	N/A	New Jersey, USA
87. *Colpodella* sp.	Cat blood	N/A	N/A	North Carolina, USA
88. *Colpodella angusta*	Mucus from *Acropora formosa*, Birch Aquarium	N/A	N/A	San Diego California, USA
89. *Colpodella angusta*	Anoxic marine sediment, Bolinas Tidal Flat	N/A	N/A	Bolinas, California, USA
90. Uncultured *Colpodella*	Intertidal thrombolites	N/A	N/A	Florida, USA
91. Uncultured *Colpodella*	Intertidal thrombolites	N/A	N/A	Florida, USA
92. Uncultured *Colpodella*	Intertidal thrombolites	N/A	N/A	Florida, USA
93. Uncultured *Colpodella*	Intertidal thrombolites	N/A	N/A	Florida, USA
94. *Colpodella* sp.	Cattle manure, identified with *Parabodo* sp.	N/A	N/A	Kansas, USA
95. *Colpodella angusta*	Soil, trembling aspen rhizosphere, elevated CO_2 conditions	N/A	N/A	Michigan, USA
96. *Colpodella* sp.	Button and pink thrombolithic mats	N/A	N/A	Bahamas
97. *Colpodella* sp.	Hypersaline Lake Tyrrell	N/A	N/A	Australia
98. *Colpodella angusta*	Wastewater	N/A	N/A	Australia
99. *Colpodella angusta*	Wastewater	N/A	N/A	Australia
100. *Colpodella angusta*	Wastewater	N/A	N/A	Australia
101. *Colpodella* sp.	Wastewater	N/A	N/A	Australia
102. *Colpodella angusta*	*Megalapteryx didinus* coprolite, sample 01098a, animal feces/manure Dart River Valley	N/A	N/A	New Zealand

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103. Colpodella sp.	Non crust habitat, Asgard Range	N/A	N/A	Antarctica
104. Colpodella angusta	Soil in front of the Brazilian Antarctic Station	N/A	N/A	Antarctica
105. Colpodella angusta	Oxygen-depleted intertidal marine sediment, upper 2 cm, Greenland	N/A	N/A	Arctic
106. Colpodella angusta	Composting diary manure	N/A	N/A	
107. Colpodella angusta	Marine	N/A	N/A	
108. Colpodella angusta	Animal feces/manure, pig manure storage pit	N/A	N/A	

1.2. Colpodella species infections in human and animal hosts

A blood infection was reported in a 57 year old woman from Yunnan Province in China with relapsing illness and an immunodeficiency of natural killer cells [22]. Giemsa staining of blood cells demonstrated intracellular infection in erythrocytes [22]. In a second case of relapsing fever reported in a male patient, *Colpodella* DNA sequences (NCBI accession number MF594625) were also identified. However no further details were provided regarding pathogenesis or treatment. A 55 year old female patient from Heilongjiang Province of Northeast China developed neurological symptoms following a tick-bite [23]. Polymerase chain reaction of DNA in blood and cerebrospinal fluid (CSF) using primers targeting *Babesia* spp. identified *Colpodella* spp. in CSF but not in the blood sample [23]. It is unclear if the tickborne infection described for the patient was zoonotic [23]. *Colpodella gonderii* along with its prey *Colpoda steinii* was identified in the urine of a 70 year old female patient with a history of chronic diseases, in Cluj-Napoca, Romania by wet-mount microscopy and Giemsa staining [26]. The patient was admitted for respiratory symptoms including breathing difficulties, dyspnea with orthopnea but no urinary symptoms. Giemsa staining identified *Colpodella gonderi* and *Colpoda steinii* trophozoites. After treatment with ceftriaxone and metronidazole, both protists were no longer detected in the urine [26]. In each of the human cases, no other parasites or pathogens were detected. In animal infections, pathology was described in a South China Tiger (*Panthera tigris amoyensis* Hilzheimer), that developed symptoms following a tick bite [12] and in pangolins heavily infested with ticks [11]. In both animal cases multiple organ failure was described upon anatomical examination and histological evaluation of damaged animal tissues [11, 12]. The ticks infesting the pangolins also contained bacterial pathogens. However, contributions to the pathology by *Colpodella* spp. is in question since the characteristics of *Colpodella* spp. pathogenesis among animal hosts has not been described or defined. What remains unclear is whether the increased reports of *Colpodella* spp. detection in ticks and animals reflect increased incidence of infection or whether the recent increased reports are due to the result of more epidemiological screenings being carried out to evaluate piroplasm and *Cryptosporidium* infections in domestic animals, live stock and wild life. It is possible that *Colpodella* spp. infections have always been occurring but have been misdiagnosed in humans and animals, maybe the hosts have been asymptomatic, and no clinical presentations manifested so there was no need to identify an infecting pathogen. Asymptomatic hosts can serve as reservoirs of infection and can pose a problem with potential infections in the elderly, susceptible hosts, immunocompromised individuals and hosts with varying immunodeficiencies. Multiple species and strains of *Colpodella* have been identified in arthropod and vertebrate hosts. Phylogenetic analysis shows a clustering of species and strains in groups related to the species and strains identified in human and animal infections showing symptoms and pathogenesis, as well as in clusters associated with specific animals and ticks [12, 18, 19, 22, 23]. The presence of the same species and

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strains of *Colpodella* spp. identified in ticks and animals and also in humans is suggestive of zoonosis, but this has to be investigated and confirmed. Microscopy demonstrated intracellular infection within erythrocytes for the human infection from Yunnan Province, China. In infected animals where blood was examined, microscopy was not performed at the time of sample collection, so that it is unclear if *Colpodella* spp. infection was intracellular or extracellular in the animal blood examined [10, 12, 24, 25, 36, 37, 38]. In a cat infection where *Colpodella* spp. was the only agent identified, *Colpodella* trophozoites or cysts were not detected in damaged tissues processed for histology and staining by hematoxylin and eosin (H&E) staining and by Giemsa stain [25]. Were life cycle stages present in the tissues and they were not recognized due to a lack of knowledge regarding the morphology of *Colpodella* trophozoite and cyst stages? It is also unclear if *Colpodella* spp. was responsible for the gastrointestinal disturbances leading to diarrhea in small ruminants, an ostrich and calves with symptoms of diarrhea [18] (Table 1). What life cycle stages of *Colpodella* spp. were present in the fecal samples? Knowledge of whether cyst or trophozoite stages are present in samples collected from arthropod and vertebrate hosts will be essential for understanding the dynamics and epidemiology of *Colpodella* spp. transmission and spread within individual hosts and among hosts, in sites of infection and for the mode of pathogenesis.

2. Is *Colpodella* species an opportunistic or zoonotic parasite?

It is unknown if all *Colpodella* species are parasitic and equally pathogenic. Therefore, arthropod competency to function as biological vectors, and the putative lack of competency in *Colpodella* spp. to enhance their parasitism needs to be investigated. Furthermore, the mode of survival of *Colpodella* spp. in the arthropod and vertebrate hosts and their source of nutrients also needs to be investigated. Due to the type of infections reported for *Colpodella* species in human and animal hosts, *Colpodella* spp. may behave like the free living amoeba that can cause highly pathogenic infections "occassionally" and have been referred to as opportunistic, accidental or amphizoic [39]. It is also unclear if *Colpodella* spp. are zoonotic. Due to the presence of *Colpodella* spp. in arthropods and the potential to infect human hosts through tick bites, *Colpodella* spp. infections pose a public health problem. Many emerging diseases currently described in humans are zoonotic [32]. Zoonotic pathogens are represented in all groups of pathogens, including viruses, bacteria, fungi, parasites and prions [32]. Mathison and Sapp [40] include *Colpodella* spp. among the eukaryotic parasites of humans. Whether *Colpodella* species are true pathogens is a serious matter as ticks carry and spread many pathogens and are capable of transmitting pathogens to humans and animals. Most significantly, and of most importance to public health is that ticks are capable of transmitting zoonotic pathogens from animals to humans [41]. From the reported cases, it appears, *Colpodella* spp. have been transmitted through ticks and as direct transmissions (Table 2). *Colpodella* species have been detected in six tick genera, a biting fly and in host tissues and body fluids (Table 2). No transmission stages for *Colpodella* species have been described in any of the reports published so far and the morphological identity of the life cycle stages found in arthropod, human and animal hosts is unknown. If we are to understand and properly manage "emerging" *colpodellosis*, then an integrated diagnostic protocol that includes staining and microscopy to identify the morphology of life cycle stages must be employed along with molecular diagnostic protocols, genotyping and immunoassays to identify stage specific antigens and markers for *Colpodella* species. Taber's medical dictionary [https://www.tabers.com/tabersonline/view/Tabers-Dictionary/736397/all/_osis (Accessed 1/29/25)] defines the suffix "-osis" as a *condition, status or process, whether normal or diseased, or sometimes an increase.* We are confronted with such a situation with the recent increased reporting of *Colpodella* spp. in animal infections, in humans and in arthropods (Table 3). This increased detection merits assigning to *Colpodella* spp. the designation of an infection, namely, "colpodellosis". A level of protocol standardization will have to be developed to ensure accurate detection and description of *Colpodella* species and strains, with a recognition of the morphology of the life cycle stages identified in the host. Such protocols are used for human parasitic infections, including infections caused by apicomplexans like

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Table 2. Arthropod hosts, tissue and body fluids containing *Colpodella* spp. associated with symptoms and pathogenesis.

Source found with *Colpodella* spp.	References
Ticks	
Ixodes persulcatus	[23]
Rhipicephalus (Boophilus) microplus	[9]
Rh. bursa	[10]
Rh. duttoni	[12]
Rh. haemaphysaloides	NCBI accession number MH208621
Haemaphysalis longicornis	[12, 14]
H. flava	[12]
H. bispinosa	[12]
H. hystricis	[12]
Hyalomma dromedarii	[13]
Dermacentor everestianus	NCBI accession number MH012047
D. nuttalli	NCBI accession number MH012045
D. andersoni	[12]
D. atrosignatus	[12]
D. taiwanensis	[12]
Amblyomma javanense	[11]
Biting fly	
Stomoxys indicus	[15]
Host tissue and body fluids	
Skin	[27]
Blood	[22, 24, 25, 36, 37, 38]
Cerebrospinal fluid	[23]
Urine	[26]
Fecal samples	[17, 18, 19], NCBI accession number JN245625

babesiosis and cryptosporidiosis, and include cell culture, staining and microscopy, nucleic acid amplification & hybridization employing polymerase chain reaction (PCR) and clustered regularly interspaced short palindromic repeats (CRISPR)-Cas (CRISPR associated protein) protocols, immunoassays and informed treatment stategies [42]. Co-infections of *Babesia* spp. and *Colpodella* spp. have been reported, thereby necessitating the need to apply similar diagnostic protocols used for babesiosis which include microscopic evaluation of thick and thin blood smears as microscopy is the gold standard for laboratory diagnosis in cases of human infection. Polymerase chain reaction, in vitro culture and xenodiagnosis in lab animal models are also used to detect different *Babesia* spp. [42]. In investigations resulting in the identification of a new species of *Babesia* known as *Babesia galileei*, Romanowsky staining was performed along with PCR using primers targeting piroplasmid 18S rRNA, cytochrome B and Heatshock protein 70 genes for amplification of DNA extracted from cat blood and salivary glands of ticks [43].

2.1. Colpodellosis in the making

What type of association exists between *Colpodella* spp. and its arthropod, human and animal hosts? Does the association meet the definition of a parasite where *Colpodella* spp. and its hosts are in a one-sided symbiotic relationship resulting in *Colpodella* spp. *benefiting at the expense of its vertebrate hosts* [44, 45, 46]? What virulence factors are present in *Colpodella* species? Olano et al. [44] describe factors involved in the evolution of virulence in pathogens and these factors include *natural selection, direct selection, coincidental evolution, short-sighted within-host selection, pathogenic manipulations of host immunity,* and *source-sink model*. It is not a premature exercise to speculate and to pose theoretical questions aimed at determining the parasitic nature of *Colpodella* spp. If the cases of single infections by *Colpodella* spp. caused the symptoms and pathogenesis described in human and animal hosts and the pathology described in animal infections, then we must investigate further to know what life

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cycle stages are present in the arthropods and the blood, CSF and fecal samples of the hosts reported. Futhermore, we must identify stages contributing to pathogenesis. What factors are contributing to the increased detection of *Colpodella* spp. in ticks, humans and animals? Is it too early to make categorizations about the virulence and pathogenicity of *Colpodella* species? Are we beginning to see dependence of *Colpodella* spp. on arthropod and vertebrate hosts, away from dependence on protist prey in the environment, or a decrease in the abundance of protist prey in the environment, due to the increased temperatures occuring as a result of climate changes across the globe? In the pathology described for the South China tiger bitten by a tick, only *Colpodella* spp. was identified in the ticks [12]. If we consider the development of virulence in this case, has transmission become "enhanced" and are the ticks and biting flies true biological vectors for *Colpodella* spp. and not mere mechanical vectors or accidental hosts? If so, what life cycle stages develop in the ticks and biting flies, and are prey organisms for *Colpodella* spp. also present in the arthropods? How is *Colpodella* spp. transported from the site of entry into the host to tissue sites within the host i.e., movement of *Colpodella* spp. from point A to point B as it were? So far there is no evidence for direct selection where the virulence of *Colpodella* spp. is directly related to its transmission rate [44]. *Colpodella* spp. have been reported in arthropods, humans, animals and birds (Table 1). It is unclear if we are dealing with host specificity among the ticks and vertebrate hosts carrying *Colpodella* spp. A vexing problem that is preventing progress in determining whether *Colpodella* spp. are true pathogens is that we do not know what life cycle stages are entering the host, establishing infection, mediating spread within the host, enhancing parasitism and pathogenesis and allowing for exit or egress of *Colpodella* spp. from the host back into the enviroinment. We need to identify the life cycle stages mediating transmission and determine if infection is intracellular or extracellar in host blood and tissues. Are *Colpodella* spp. in arthropods and vertebrate hosts accidental with no benefit for *Colpodella* spp. but detrimental to the host in the "right" circumstance of host susceptibility due to immunosupression or immunodeficiency? Again, we currently have no answers, but we have the opportunity to find out as *Colpodella* spp. continue to be detected in different vertebrate hosts and in different tick species, increasing the potential for zoonotic infections.

2.2. From free-living predators to opportunistic parasites

Opportunistic human infections by free-living protists are not without precedent. The thermotolerant *Naegleria fowleri* is a free-living bi-flagellate amoeba consisting of a cyst, amoeboid and flagellated trophozoite stages found in the soil and in ponds, lakes, and other aquatic environments, including some tap water [47, 48, 49, 50]. When water contaminated with *N. fowleri* is forced into the nasal cavities through activities like diving or splashing of water in lakes or contaminated pools, *N. fowleri* can penetrate the cribriform plate and enter the brain where it causes primary meningococcal encephalitis (PAM) [39, 47, 51, 52]. Understanding differential gene expression in life cycle stages of parasites is critical to identifying genes essential for transmission, invasion, virulence, pathogenesis and for metabolic adaptations in the environment, in vectors and in host tissue. These types of investigations are ongoing to understand infections caused by *N. fowleri* and other opportunistic free-living parasites [51]. Such an approach will be necessary to understand parasitism and pathogenesis by *Colpodella* spp. *Acanthamoeba* spp. are also free-living protists, and part of a group of free-living amoeba (FLA) along with *Naegleria fowleri*, *Balamuthia mandrillaris, Sappinia* spp. and *Vermamoeba vermiformis* that have trophozoite and cyst stages in their life cycles [39, 53, 54, 55, 56, 57, 58] and cause acute life-threathening infections as opportunistic parasites [39, 59]. Among the three most studied FLA, high mortality rates result from infection with *B. mandrillaris* at 90 %, *Acanthamoeba* at 98 % and *N. fowleri* at 99% [39]. Among forty-seven species of *Naegleria* known, only *N. fowleri* causes fatal PAM infections [51]. However, there are several strains of *N. fowleri* resulting in investigations to identify virulent strains. Identification of the thermotolerant *Naegleria* species such as *N. australiensis* in water samples represents an indicator for the presence of the more serious *N. fowleri* in environmental, wastewater and tap water samples [49, 55]. *Acanthamoeba* spp. cause serious diseases like granulomatous inflammation, keratitis, encephalitis and primary

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meningitis in humans [53, 54]. Infections can become severe in immunocompromised individuals where disseminated infections can occur [54]. In the FLA, an integrated approach for diagnosis is used for identification and includes staining, microscopy, cell culture, nucleic acid amplification and immunoassays [52, 59, 60, 61]. Different strains of *Acanthamoeba* cause different levels of infection severity, pathogenesis and tissue sites infected in the host [58]. Genotyping has aided epidemiological investigations and transmission dynamics of FLA and piroplasms to identify potential hosts at risk for infection, resulting in protocols that provide preventive measures for public safety [50, 52]. As more cases of colpodellosis caused by single or coinfections are reported, diagnosis of infection will require an integrated approach to accurately identify *Colpodella* spp. present in arthropods and host specimens. The application of multiplex real-time PCR (MPL-rPCR) in combination with microscopy and sequencing was instrumental in diagniosing PAM [52], and the use of a combination of molecular techniques such as PCR, phylogenetic analysis, microscopic examination of wet mounts and trichrome staining was useful in identifying *Naegleria* species in water samples investigated in a Sri Lankan study [62]. Identification of the flagellated trophozoite stage of *N. fowleri* in CSF by microscopy provided important information regarding the morphological form of the parasite present during infection and aided in treatment [62].

2.3. Pathogenic protists are vectors for other pathogens

Acanthamoeba spp. and other amoebas act as reservoirs, vectors and hosts for bacteria, viruses, other protists and fungi [54, 63, 64, 65]. Infection with FLA exposes the human host to other pathogens contained within the amoeba, enhances lateral gene transfer among pathogens and recombination of virulence genes among bacteria and amoeba and impacts the development of antibiotic resistance among bacteria [39, 54, 63, 64]. This is an important consideration for *Colpodella* species transmitted by ticks to animal and human hosts and in direct non-tick mediated *Colpodella* spp. infections. *Colpodella* spp. may harbor pathogens obtained from bacteriovorous prey that enhance infectivity and virulence within hosts during coinfections and single infections. There is no evidence presently to indicate that *Colpodella* spp. are host to protist, bacterial, viral and fungal pathogens but it is another important factor that must be considered as the pathogenic nature of *Colpodella* spp. is explored. Screening for the presence of bodonid, ciliate or algal prey in ticks and animals that harbor *Colpodella* spp. will be important to demonstrate if a *coincidental evolution model* is at play where virulence factors developed in *Colpodella* spp. are not required solely for host parasitism but may have other advantages for *Colpodella* spp. within ecosystems in the environment [44]. In the cases where pathogenesis has been reported, strains of *Colpodella* species identified in humans have also been identified in ticks and in the animals showing tissue pathology, suggestive of the development or presence of virulent strains of *Colpodella* spp. and a selective mechanism at work that may enhance zoonosis [44]. However, *Colpodella* spp. strains identified in human and animal infections have also been described in various geographic environmental locations globally, from different tick species in different environments and in environmental samples such as from soil, dead leaves and aqueous sources (Table 1). This is in contrast to what would be expected in the *short-sighted within-host evolution* model for virulence proposed by Olano et al. [44]. There are no human deaths reported from colpodellosis and so far there is no evidence of host selection for any of the *Colpodella* species and strains among the many that have been identified by PCR and DNA sequencing. Death was reported for the South China Tiger and pangolins, although the contributions to pathogenesis by *Colpodella* spp. in the pangolins is unknown [11]. Similarly, severe tissue damage was reported in a domestic cat with putative *Colpodella* spp. infection [25]. There is also no evidence of host site selection or site preferences by *Colpodella* spp. once inside human and animal hosts, so we cannot consider site location changes within the host by *Colpodella* spp. that will lead to more fulminant and lethal infections within the host. If such a scenario is possible within human and animal hosts we must investigate it, in the likelihood that *Colpodella* spp. transmitted by arthropods from animal to human hosts, as a zoonosis, may support this model.

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2.4. Culturing Colpodella species for identification of virulence markers

In previous studies we identified exon 7 of the RhopH3 gene, that encodes the RhopH3 rhoptry protein in *Colpodella* sp. ATCC 50594 using primers targeting *Plasmodium falcipa*rum RhopH3 [66]. We recently identified the Kelch 13 and coronin genes in *Colpodella* sp. ATCC 50594 using oligonucleotide primers targeting the Kelch 13 and coronin genes in *P. falciparum* [33]. Both genes encode proteins that function in endocytosis and serve as markers for endocytosis in *P. falciparum* [67, 68, 69, 70, 71, 72, 73, 74]. As reports of colpodellosis continues to increase, important antigens recognized by the host immune system would need to be identified. Human cases reporting blood infections, with an intracellular location for *Colpodella* spp. within erythrocytes, and *Colpodella* spp. in CSF and urine have been described [22, 23, 26, NCBI accession number MF594625]. Are these the only known infection sites within the body where *Colpodella* is found? Blood samples collected during screenings of domestic animals, livestock and wildlife may contain antibodies to *Colpodella* spp. antigens and should be checked using immunoassays. This will mean that the samples containing *Colpodella* spp. will need to be cultured to obtain pellets for protein extractions. In the cases where the same *Colpodella* spp. strains are identified in humans, ticks, flies and animals, does this represent an ecological switch of habitats representative of the *"source-sink" model* of Olano et al. [44] where virulence evolution is taking place as *Colpodella* species move from the environment, through arthropod hosts, into human and animal hosts and back into the environment in a parsitic life cycle that still needs to be confirmed? The life cycle of *Colpodella* sp. ATCC 50594 in culture has been described [7, 8] and the consistency of the life cycle in culture allows for experimental design and planning, which has led to ultrastructural studies that identified how *Colpodella* sp. ATCC 50594 aspirates cytoplasmic contents of the prey during myzocytosis [7, 75] and revealed previously unknown developmental stages of *Colpodella* spp. Knowledge of the life cycle in diprotist culture also allowed investigations of nutrient uptake using nanometer beads [35]. The presence of *Colpodella* spp. in a diprotist culture with *Parabodo caudatus* poses a challenge to investigations since culture collections for protein and nucleic acid extractions contain macromolecules from both protists. However, *P. caudatus* can grow in a monoprotist culture in *Enterobacter aerogenes* bacterized Hay medium, and so extractions of protein and nucleic acids are also performed from the monoprotist culture to provide controls in experiments. Both protists have distinct morphological forms based on the difference in their taxonomic position. *Colpodella* spp. are related to the Apicomplexans and have many features found in the apicomplexa including an apical complex and conserved genes found in the apicomplexa and *P. caudatus* is a kinetoplastid with the trophozoites possessing a nucleus and kinetoplast (mitochondrion) that can be distinguished by staining and microscopy [8]. Hay medium is currently used to culture *Colpodella* sp. ATCC 50594 allowing for large volumes of culture to be obtained for protein, DNA and RNA extraction. A major advance that will benefit future investigations of *Colpodella* spp. detected and isolated from arthropods, human and animal hosts will be the development of alternate culture systems to culture *Colpodella* spp. axenically in a monoprotist culture. The current diprotist culture conditions in Hay medium will aid in the identification of additional genes of *Colpodella* spp. and the verification of gene conservation as we have shown in our studies [33]. Moreover, the function of encoded proteins can be investigated during transmission, establishment of infection, and pathogenesis. Encystation and excystation can be investigated to better understand the life cycle of *Colpodella* spp. Immune response to *Colpodella* spp. antigens, and the mechanisms of *Colpodella* spp. exit from the host can be investigated in culture and in animal models to gain a better understanding of disease processes in colpodellosis.

3. Techniques for detecting life cycle stage markers of *Colpodella* spp. in arthropod and vertebrate hosts

Among the apicomplexa, parasites are found in different tissues of the body in both humans and animals. The hemosporidians parasitize intracellular locations, being found within the erythrocyte for *Plasmodium* spp., *Babesia* spp., *Haemoproteus* spp., in lymphocytes for *Theileria* spp., in

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macrophages and other nucleated cells such as epithelial cells for *Toxoplasma gondii* and muscle cells for *Sarcocystis* spp. *Cryptosporidium* spp., *Cyclospora* spp. and *Eimeria* spp. infect epithelial cells of the gastrointestinal tract [45]. Among the intracellular infections within erythrocytes and lymphocytes, parasites remain within a parasitophorous vacuole (PV) that is formed during host cell invasion or they can exit the PV and develop directly within the cytoplasm. Pathogenic protists including amoeba, flagellates and ciliates range from opportunistic pathogens like *Naegleria fowleri* and *Acanthoameba castellenii* [45] to frank zoonotic pathogens like *Babesia* spp., and pathogens like *Plasmodium* spp., *Entamoeba histolytica*, *Giardia lamblia*, and *Trichomonals vaginalis* [45]. Sites for pathogenesis range from the brain for *N. fowleri*, eyes for *A. castelleni*, and GI tract, skin, brain, liver for *E. histolytica*. It is not unprecedented for free-living protists to become opportunistic and cause severe acute infections as is the case for the biflagellated *N. fowleri*, whose flagellated trophozoite stage initiates infection when forced into the nasal cavities and through the cribriform plate to the brain [39, 45].

3.1. Mechanisms of pathogenesis in colpodellosis. What do we know?

For *Colpodella* spp., we need to be able to describe whether trophozoites attach to host cells and express ligands used for adhesion or attachment to receptors on host cells. If cysts are present in the hosts, we need to know if cyst molecules promote inflammatory responses and whether the molecules stimulate innate and adaptive immune responses. If *Colpodella* spp. trophozoites penetrate host cells, we need to be able to describe specific host cells, receptors expressed on those cells and the participation of apical complex organelle contents from the rhoptries or micronemes acting as ligands for the host cell receptors. Whether endocytosis or phagocytosis is essential for intracellular invasion of host cells would need to be confirmed. Furthermore, *Colpodella* spp. proteins facilitating establishment of infection and nutrient acquisition would also need to be described. Modes of nutrient uptake such as endocytosis, phagocytosis, myzocytosis and trogocytosis which can be used to obtain nutrients within the host must be described. Pathogenic protists like *Entamoeba histolytica* and *Trichomonas vaginalis* feed by trogocytosis which mediates pathogenesis during infection [76, 77]. The mechanism of *Colpodella* spp. multiplication within the host and spread to other tissues, as well as the development of cysts, if this occurs would need to be demonstrated. Presently, none of this is known. *Colpodella* spp. have been described in blood, CSF, urine and fecal samples and on the skin [22, 23, 24, 25, 26, 27] (Table 1). Direct damage to host tissue can occur because of physical destruction of the host tissues by parasites physically destroying host cells, inducing inflammation or apoptosis, secretion of proteolytic enzymes, induction of cytokine secretion, or immune mediated damage due to antibodies, cytotoxicity, or cytokines produced in response to the parasitic activities of *Colpodella* spp. Whether *Colpodella* spp. are true pathogens remains a critical question that needs to be answered. The mode of nutrient uptake within the host, the spread of life cycle stages within the host and markers of virulence, hold clues for the mechanisms of pathogenesis in the host [78]. In order to fully understand how to prioritize efforts to investigate the biology of *Colpodella* spp. and identify additional molecular markers that will aid molecular diagnostic methods, identify host sites where *Colpodella* resides along with the life cycle stages present within the host, sample sources containing *Colpodella* spp. will need to be cultured, stained for microscopy and the morphology of *Colpodella* spp. described. Antibodies specific for *Colpodella* spp. life cycle stages will need to be identified. In animals shown to be positive for *Colpodella* spp., by PCR, serum samples from the animals can be collected to screen for *Colpodella* spp. proteins following short-term in vitro culture of samples such as hemolymph from ticks, blood, CSF and fecal samples from hosts as these samples may contain *Colpodella* spp. along with their prey. It will also be important to detect prey organisms for *Colpodella* spp. in the examined samples. *Parabodo* and *Bodo* spp. were identified with *Colpodella* spp. in cattle manure [20] and in blood samples of dogs [37] suggesting that prey species for *Colpodella* spp. may also be present in arthropod, human and animal specimens screened for *Colpodella* spp. *Parabodo caudatus* was identified in dog urine [79]. Western blotting can be used to identify proteins reactive with serum antibodies from infected humans and animals. Human antiserum from the female patient

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with relapsing fever was used in an immunofluorescence assay (IFA) to localize *Colpodella* spp. proteins within the erythrocyte [22].

3.2. Microscopy: The gold standard for parasite identification

Contrary to statements that declare microscopy to be unimportant or unecessary for detecting *Colpodella* life stages, being able to identify the morphology of infectious disease organisms has been a fundamental feature of infectious disease, parasite investigations and diagnosis [44, 80]. At the same time that diagnostic methods are relying more on digitized molecular platforms and less on morphological methods and microscopy, the number of trained microscopists and morphologists in parasitology is declining [80]. In the emergence or re-emergence of infectious disease organisms world-wide, a shortage of trained microscopists is a tremendous set back for public health [80]. Lack of expertise and inadequate morphological identification of parasites in general and emerging parasites like *Colpodella* spp. in particular can lead to misidentification of parasites. Failure to identify parasites in situations where molecular techniques are inadequate for the sample being tested like in fecal samples may lead to delayed diagnosis. Lack of sufficient genomic, proteomic and transcriptomic data in the databases for organisms like *Colpodella* spp. and other organisms that are not available in the databases can negatively impact the progress needed to understand the disease causing characteristics of *Colpodella* spp. [80, 81, 82]. Additionally, delays in diagnosis and worsening conditions due to delays in appropriate treatment will be detrimental to patient outcomes. For colpodellosis, morphological identification of the protist from collected samples is crucial for identifying life cycle stages initiating transmission and responsible for pathgenesis. In resource poor settings, expensive equipment for molecular testing may not be available, may be expensive and the wait times to receive results from samples submitted to reference labs may result in delays in patient diagnosis. *Colpodella* spp. have been identified in a variety of global locations and in animals in close contact with humans. PCR may not be available in some settings to make a molecular diagnosis. An integrated diagnostic approach that includes morphological identification of stained pathogens along with serology and molecular identification of DNA is more comprehensive and suitable for reliable identification of pathogens and will provide accurate diagnosis of infections in patients, particularly in identifying life cycle stages within the host [41]. Prevention efforts will be futile if the life cycle stage to target is unknown. The following techniques demonstrate the utility of integrating different diagnostic approaches to identify pathogens (Figure 1).

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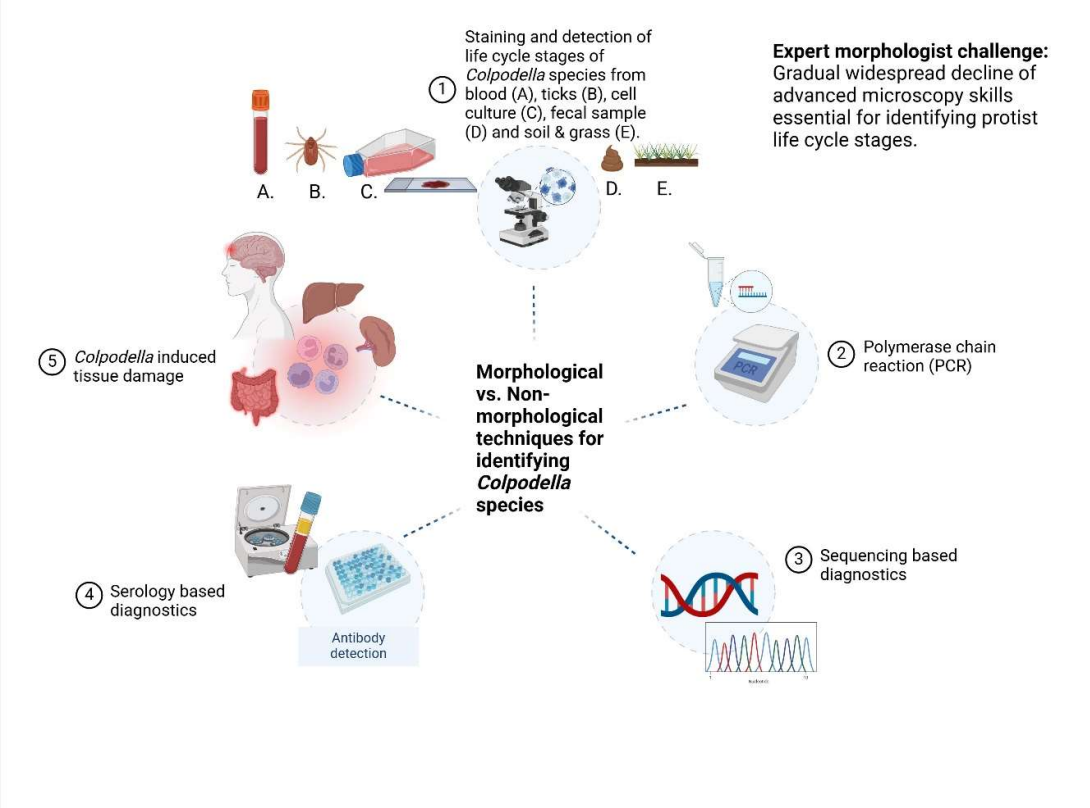

Figure 1. Techniques for identifying *Colpodella* species in arthropod, human and animal species and in the environment. A, blood, B, ticks, C, culture, D, fecal samples and E, soil and grass. *Colpodella* spp. can be formalin fixed, smeared on glass slides for staining and microscopy. The figure was created using BioRender.com (accessed 1/4/ 2025).

3.3. Morphology-based diagnostic techniques

Morphology-based techniques utilizing staining of fixed smears prepared from blood, CSF and other body fluids as well as processing of fixed and sectioned tissue for histology needs to be performed to identify life cycle stages of *Colpodella* spp. Life cycle stages of parasites and other infectious disease organisms can be detected using these approaches. Cell culture of parasites to increase cell density for observations or propagation of the parasites in animal models for morphological evaluation also help confirm the identity of pathogens. This is currently a missing piece in the investigations of *Colpodella* species. Identifying the morphology of life cycle stages and understanding life cycle stage transitions within the host is essential for determining transmission stage dynamics, the infection's progression and severity, calculating parasite density in the bloodstream and other body fluids, and distinguishing the infection from other diseases with similar symptoms. In the reported cases of *Colpodella* spp. infections, the specific life cycle stages responsible for transmission and pathogenicity remain unknown.

3.4. Parasite Induced Tissue Damage

Colpodella spp. has been reported to cause inflammation and neurological symptoms in a tickborne human case of *Colpodella* infection [23]. An immunosupressed female patient with natural killer cell deficiency had intracellular red blood cell infection with *Colpodella* spp. along with relapsing fever and a male patient also reported to have relapsing fever was shown to have *Colpodella* spp. infection [22, NCBI accession number MF594625]. In the three cases, only *Colpodella* species was identified by PCR amplification of the 18S rRNA gene. The role of *Colpodella* spp. and any secreted/excreted products contributing to symptoms and pathogenesis in the patients is unknown.

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Colpodella gonderii and its prey *Colpoda steinii* were detected in the urine of a female patient experiencing chronic diseases along with a urinary tract infection. Treatment resolved the infection leading to the clearance of both protists [26]. However, it is unknown if the protists contributed to the infection. Giemsa staining was used to identify the morphology of predator and prey in the urine sample. It is unclear which type of tissues in the host might be most affected by *Colpodella* spp. Morphology-based diagnostics are important for assessing parasite-induced tissue damage. In animal infections, a South China tiger developed extensive tissue damage following symptoms that developed after a tick bite containing *Colpodella* spp. No other pathogen was reported in the tiger's infection [12]. It is possible that *Colpodella* spp. by itself can induce immune related pathogenesis or by itself cause tissue damage through the secretion of enzymes and other molecules that promote tissue invasion and destruction. Because life cycle stages have not been identified by microscopy at specific tissue locations, it is unclear how tissues and organs were damaged in the animal host [12] and how *Colpodella* spp. infection induced the inflammation and neurological symptoms observed in human and animal infections.

3.5. Expert morphologist challenge

Increased reliance on non morphology-based detection technologies has led to the gradual, widespread decline of advanced microscopy skills essential for identifying parasite morphology and maintaining morphology expertise in the field. In a period when public health is threathened by emerging and re-emerging diseases, the loss of microscopy expertise is detrimental to accurate parasite and infectious disease agent recognition and identification [80]. Consequently, the objectivity of results remains a limitation due to the need for expert microscopists, parasitologists and pathologists who understand the significance of parasite life cycles in aiding accurate diagnosis. Several studies have evaluated the ultrastructure of different *Colpodella* spp. [2, 3, 4, 6, 7]. Phase contrast microscopy, DIC microscopy and scanning electron microscopy (SEM) have also been applied to examine the morphology of different *Colpodella* spp. [2, 7, 8, 83]. These types of microscopy require expensive equipment and highly technical expertise along with long times for processing samples, and viewing and capturing images. Light microscopy remains a gold standard for identifying many parasites even in the advent of advanced molecular protocols [84]. This must be applied to *Colpodella* spp. investigations to aid morphological characterization of *Colpodella* spp. life cycle stages. We have evaluated traditional staining techniques like Giemsa staining, Wheatley's trichrome staining and Kinyoun's staining to identify trophozoites and cysts of *Colpodella* sp. ATCC 50594 [85]. The traditional staining techniques could allow for identification of trophozoites but were inadequate for distinguishing different developmental stages of *Colpodella* cysts and differentiating cysts of *Colpodella* from cysts of the prey, *P. caudatus*. We developed a new staining protocol, Sam-Yellowe's trichrome staining that identifies trophozoites, different cyst stages and clearly distinguishes *Colpodella* sp. ATCC 50594 cysts from *P. caudatus* cysts [7, 85]. The new staining protocols will be useful in identifying *Colpodella* species present in specimens collected from arthropods, human and animal hosts.

3.6. Polymerase Chain Reaction (PCR) and Sequencing Based Diagnostics

PCR techniques offer high sensitivity, enabling the identification of very small amounts of parasite DNA, which is crucial in scenarios where low levels of parasites in subclinical infections may pose a risk of reactivation or when dealing with cases of asymptomatic infections. Additionally, when the parasite levels are low in clinical samples, isolation of parasites for culture and detection through microscopic examination of smears can be challenging. The availability of different staining protocols, the processing of blood smears as thick or thin smears and the use of different fixation methods has been instrumental in maintaining microscopy as a central technique aiding accurate parasite diagnosis. In order to fully understand the epidemiology and distribution of species and strains of *Colpodella* spp., samples containing *Colpodella* spp. must be cultured and stained in addition to extracting DNA for PCR or RNA for qPCR. Among the pathogenic apicomplexa, organisms like

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Plasmodium have distinct morphological differences among the human, animal and avian species [86, 87, 88]. Morphological changes yet unknown may occur once *Colpodella* spp. is present in arthropod, and vertebrate host tissue. If this is the case, we must identify these life cycle stages to aid rational diagnosis and treatment plans. Similarly, distinct morphological differences are seen among different *Babesia* species [45, 89]. Morphological differences also exist among the known *Colpodella* speices [2, 3, 4, 6, 7]. *Colpodella* species also have different morphological appearances and different sizes. For example DIC images show trophozoites of *C. pugnax* Cienkowsky, 1865 to be thin, fusiform and 7-15 μm in length with a prominent curved rostrum [2]. *Colpodella turpis* has a trophozoite that is 14-22 μm in length with a wide body, large hooked rostrum and flagella of equal length [2]. Unfed *Colpodella gonderi* trophozoites are as small as 5 μm, without a prominent rostrum and two flagella that can be two or more times longer than the body [2]. Trophozoites of *Colpodella* sp. ATCC 50594 are fusiform shaped, 7-9 μm in length with a slightly hooked rostrum. Morphological differences as well as structural differences observed ultrastructurally contributed to the change in the genus designation of *Colpodella pontica* to *Voromonas pontica* [6]. These critical differences underscore the need to culture and stain *Colpodella* spp. identified in arthropods, animal and human infections. In PCR reactions using primers targeting genes from pathogenic apicomplexans, nested PCR reactions along with DNA sequencing of amplicons have helped in the detection of *Colpodella* spp. However, PCR tests are not readily available in resource-poor settings where *Colpodella* spp. might be causing infections along side known apicomplexan, bacterial and viral infections. Sequencing-based diagnostics have an advantage over both morphology-based and non morphology-based methods due to its ability to provide species level identification and geographic strain differentiation. Notwithstanding, the morphology of *Colpodella* spp. must be identified in specimens collected from arthropod and vertebrate hosts.

3.7. Serology based diagnostics

Serology-based tests are useful for rapid detection of antigens and are widely employed to diagnose other Apicomplexan parasites. However, no serology-based testing techniques are available for detecting *Colpodella* spp. in clinical samples. In previous studies, we showed that antisera containing antibodies specific to *P. falciparum* rhoptry proteins and Kelch 13 proteins cross-reacted with *Colpodella* sp. ATCC 50594 antigens in immunofluorescence and western blotting [8, 33]. These data along with PCR amplification and sequencing of the genes encoding the proteins demonstrate that rhoptry and Kelch 13 genes are conserved in *Colpodella* spp. Transcriptomic analysis identified many apicomplexan genes that are found in the chrompodellids which consists of *Chromera velia*, *Vitrella brassicaformis*, *Voromonas pontica* and *Colpodella* spp. [16]. The use of serological assays to identify host antibodies and parasite antigens is routinely performed as part of integrated approaches to diagnose parasitic infection and identify etiological agents for infection. Immunoassays like enzyme-linked immunosorbent assays (ELISA) and western blotting are used to screen for antibodies reactive with parasite antigens in infections including opportunistic and zoonotic infections. Human and animal host antisera has been used to detect antigens in various body fluids and samples such as in blood, urine, fecal and CSF samples. Immunofluorescence and immunoelectron microsopy allow for the visualization of parasite antigen distribution in parasites. Antibodies against *Colpodella* spp. antigens will need to be produced and antisera from human and animal hosts will need to be collected and used to screen for the presence of *Colpodella* antigens, and allow for additional *Colpodella* genes to be identified to aid phylogenetic analysis in addition to the 18S rRNA gene currently used in investigations. *Colpodella* spp., pellets can be obtained from Hay medium cultures for use in protein extraction for immunoassays. Evaluation of antibodies to *N. fowleri* in a Houston-Galvaston Texas (USA) healthy population found 89% seropositivity to *N. fowleri* [59]. In a study performed in northwestern Mexico, antibodies to the FLA, *N. fowleri*, *Acanthamoeba* and *Balamuthia* were identified in asymptomatic children and adults [90]. Immunoglobulin (Ig) M and IgA antibodies have also been detected in saliva with higher titers obtained in individuals with upper respiratory tract infections [90]. With the ubiquity of *Colpodella* spp. as indicated by the global distribution that has now emerged,

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more humans and animals may be exposed to *Colpodella* spp. than is currently understood. Serological assays as an intergrated component of epidemiological screening and clinical diagnosis cannot be ignored.

3.8. Cost Effectiveness

Diagnostic methods in parasitology consist of microscopy, immunoassays and molecular biology techniques. Each techniques varies in the level of sophistication, equipment cost and the need for highly skilled laboratory personnel. These techniques are used in integrated approaches to identify parasite stages in blood, CSF, urine, sputum, human and animal tissues and fecal samples. Parasites in wet mounts, stained smears and in histological samples can be identified by microscopy which remains the gold standard for parasite detection even though sensitivity may be weaker than that of immunoassays and nucleic acid amplification protocols [84]. Two major advantages of microscopy include the identification of different life cycle stage of parasites following staining and the simultaneous identification of multiple parasite species in one smear [84]. For example, among apicomplexans different species and life cycle stages of *Plasmodium* spp., *Babesia* spp., and *Theilaria* spp. can be identified by microscopy. Blood and sexual stage differences of *Plasmodium* species are a standard feature of malaria diagnosis [https://www.cdc.gov/dpdx/malaria/index.html accessed 2/10/25]. The detection of *Colpodella* spp. from arthropod, human and animal hosts will greatly benefit from microscopy since no life cycle stages have been identified in any of the cases of animal and human colpodellosis reported in the literature. Different life cycle stages may be present in arthropod and vertebrate hosts. Such differences are recognized in *Plasmodium* spp. where different stages such as sporozoites, oocysts and ookinetes are present in the female *Anopheles* mosquito and trophozoites and gametocytes are present in the vertebrate host. Light and electron microscopy can differentiate these differences [86, 91]. In *Babesia* spp. different life cycle stages are present in the tick and vertebrate host. There is a pressing need to identify the different *Colpodella* spp. that have been detected in human and animal blood, feces, CSF, ticks and a biting fly. Many of these newly detected *Colpodella* spp. represent new species and strains of *Colpodella* for which additional investigations are needed to identify virulent and zoonotic species and strains. Some are species that have been described previously such as *C. gonderi*, *C. tetrahymenae* and *C. edax* [2, 3, 4, 5, 6, 34]. However, novel species and strains may also be present in the recent reports. Compared to molecular and immunology techniques with higher sensitivity and specificity, the use of light microscopy is less costly but requires highly skilled microscopists to correctly identify parasites [80, 92]. High equipment costs, technically trained staff and reliable infrastructure is a requirement for immunoassays and nucleic acid amplification protocols. These protocols require lengthy sample preparation methods and lengthy times to obtain results which can delay diagnosis. Point-of-care testing (POCT) is widely used for infectious disease and parasite identification and many POCT employ antibody detection of antigens from pathogens using lateral flow of reagents to result in antigen-antibody interactions in POCT devices [93, 41, 84]. The most widely used immunoassays for parasite antigen detection include ELISA, western blotting and immunofluorescent assay (IFA). Assay specificity and sensitivity is high, although problems with cross-reactivities and false negative and false positive results can occur [41, 59]. These are mitigated by the use of controls and stringencies in assay development. Molecular techniques using nucleic acid amplification protocols for DNA and RNA, used for parasite detection include PCR, quantitative real-time PCR (qPCR), digital PCR (dPCR), loop-mediated isothermal amplification (LAMP) and CRISPR/Cas12a [84]. These protocols require reliable infrastructure, costly equipment and skilled personnel to perform and interprete assay results. Combined PCR-ELISA protocols are also in use with different mutiplex assays under development for multiple antigen detection assays. 18s rRNA gene sequences, tandem repeat sequences, internal transcribed spacer (ITS) sequences and mitochondrial genes are used in nucleic acid amplification protocols [84]. With no standardized lab tests yet for *Colpodella* species detection, and with *Colpodella* spp. detected in fecal samples, blood and CSF, nucleic acid amplification tests will need to be performed from nucleic acids extracted from appropriately stored and handled samples in order to obtain useful data. Contreras-Ferro et al. [41]

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indicate that qPCR is most useful if performed within 5-10 days of symptom onset. The incubation period for colpodellosis is unknown. For many tickborne pathogens the incubation period and time for development of symptoms is known. Once these characteristics have been determined for *Colpodella* spp. it will be crucial to determine this for colpodellosis when other tickborne pathogens are identified due to the co-infections that have been reported with bacteria and the piroplasms *Babesia* spp. and *Theileria* spp., and *Cryptosporidium* spp. Amplification of 18S rRNA gene sequences have been used in most of the human and animal cases reporting *Colpodella* spp. detection. It is critical that additional genes be identified in *Colpodella* spp. to aid accurate identification of *Colpodella* species that may be missed when 18S rRNA gene sequences from piroplasms or *Cryptosporidium* spp. are used for detection. The availability of Hay medium for culturing *Colpodella* spp. and staining protocols that can be performed in less than 10 minutes will allow for *Colpodella* spp. life cycle stages to be identified, and pellets for *Colpodella* protein and nucleic acid extractions to be collected. Additionally, primers targeting the 18S rRNA genes of bodonid, algae and ciliate prey should also be used for amplification to determine if the prey are present in the arthropod, human and animal hosts. Basic light microscopy and morphology-based detection of parasites is rapid, cost effective, and does not require advanced technologies, costly equipment and higly skilled professional staff, which is beneficial in resource poor settings in low and middle income countries (LMICs) [59, 94]. Transmission of *Colpodella* spp. as a zoonotic or opportunistic parasite along with neglected parasites of poverty in endemic areas, poses an additional burden to poor communities. In the absence of costly molecular biology and immunoassay equipment, *Colpodella* spp. cannot be detected. At minimum, microscopy should be performed on collected samples to aid morphological identification of *Colpodella* spp.

4. Culture conditions for Colpodellids and chromerids

Efforts to cultivate *Colpodella* spp. along with staining for morphological identification is needed to aid molecular investigations aimed at identifying molecular markers of *Colpodella* spp. that facilitate transmission, survival and pathogenesis in hosts, and determining which species to prioritize for the risk of zoonotic infections to humans and for infections in animals (domestic and live stock, recreational animals and wildlife) [95]. The chromerids *Chromera velia* and *Vitrella brassicaformis* are included in this section because some of the same culture media used for colpodellids has been used for chromerids.

4.1. Culture media for cultivating Colpodella species

Colpodella spp. can be cultured in various types of media to allow for cell biology, molecular biology and microscopy investigations. Detection of *Colpodella* spp. genes and antigens is highly feasible, as large volumes of culture can be obtained for protein and nucleic acid extraction, and for antibody production. The availability of reliable and consistent culture conditions will aid the idetification of *Colpodella* spp. antigens that will be crucial for developing diagnostic tests. The availability of reliable culturing techniques allowed us to investigate and describe the life cycle of *Colpodella* sp. ATCC 50594 in culture [8]. *Colpodella pseudoedax* and *C. unguis* have been cultured in Pratt medium and Schmaltz-Pratt medium consisting of 1L H_2O, 28.15 g NaCl, 0.67g KCl, 5.51g $MgCl_2$ $6H_2O$, 6.92g $MgSO_4$ $7H_2O$, 1.45g CaCl $2H_2O$, 0.1g KNO_3, 0.01g K_2HPO_4 $3H_2O$), 20-22% salinity with protist prey [1]. *Spumella* sp. and *Procryptobia sorokini* served as prey in the medium. *Voromonas pontica* (formerly *Colpodella pontica*) isolated from the coastal waters of the Black Sea has also been cultured in Schmaltz-Pratt medium [2] with *Escherichia coli* and *Bodo sorokini* provided in the medium as a nutrient source for the prey. The bacteria serve as food for the protist prey and *B. sorokini* is the prey for *V. pontica*. We have cultured *V. pontica* successfully in concentrated Hay medium (VWR, Inc.) mixed with sea water, bacterized with *Enterobacter aerogenes* and cultured at 24°C with *Percolomonas cosmopolitus* as prey [96].

Colpodella gonderi isolated from a grassland site, in shallow brown soil with poor minerals was cultured in soil mixed with rainwater in a petri dish with incubation at 15°C for 4 days [34]. *Colpoda*

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steinii, *Grossglockneria acuta* and *Pseudoplatyophora nana* served as prey in the culture. *Colpodella vorax* isolated from pond water that had dead leaves and grass was cultured in pond water contained in a petri dish [3] with *Bodo caudatus*, *Spumella* sp., *Synura peterseni*, *Chilomonas paramaecium*, *Euglena gracilis* and *Colpoda cucullus* as prey for *C. vorax*.

Colpodella pugnax Cienkowski, 1865 isolated from an artificial hypersaline (26% NaCl) lagoon at Whyalla, South Australia was cultured with the prey species *Dunaliella virdis* at 28°C under varying light conditions [2]. The type of medium used for cultivation was not specified. Similarly, no culture conditions were specified for *C. angusta*. However, *Procryptobia sorokini*, *Spumella* sp. and *Parabodo caudatus* served as prey for *C. angusta* [16].

Colpodella tetrahymenae isolated from the rainforest soil from La Selva, Costa Rica was cultured with soil mixed with natural bacteria with the prey *Tetrahymena pyriformis* [16]. The medium used for suspending the soil was not specified.

Colpodella sp. ATCC 50594 is cultured in ATCC culture medium 802 (Sonneborn's Paramecium medium) [https://www.atcc.org/products/50594 accessed 2/10/25] and in Hay medium bacterized with *Enterobacter aerogenes* and *Parabodo caudatus* present in the diprotist culture as prey [66]. Hay medium is useful for growing large volumes of diprotist and monoprotist cultures in tissue culture flasks. Cultures in tissue culture flasks are examined under an inverted microscope to monitor cell growth. Bacterized Hay medium cultured overnight at 37°C is used for the subculture of *Colpodella* sp. cysts the following day. Following *Colpodella* sp. cyst inoculation of the bacterized medium, cultures are incubated at 24°C for up to 36 h post subculture [8]. Active cultures containing many young active trophozoites feeding by myzocytosis, producing transient cysts which excyst to produce juvenile trophozoites can be found within 20 to 30 h post subculture, with resting cysts beginning to form 30 to 36 h post subculture [8]. Resting cysts can remain in culture up to 14 days and can be used to initiate a new round of cultures. Hay medium can be purchased as a 1x working medium ready to be used or a concentrated stock medium can be purchased and used after diluting it with sterile distilled water to obtain a working medium [8, 96]. Centrifugation of the culture allows for the collection of pellets for macromolecular extractions. Protists in the cultures can also be fixed using a final concentration of 5% formalin, centrifuged and pellets washed and resuspended for the preparation of smears and staining for light microscopy, fluorescent staining, immunofluorescence and confocal microscopy [7, 75]. In many of the environmental sites where *Colpodella* spp. have been detected, chromerids such as *Chromera velia* and *Viterella brassicaformis* have also been detected [16, 97, 98]. *Chromera velia* was first isolated from stony coral *Pleslastrea versipora* in Australia [97] but it has also been obtained from Boothbay Harbour, ME from the culture collection of marine phytoplankton and cultivated in f/2 medium in sea water with a 12/12 or 8/16 hour light and dark cycle at 26°C [97]. *Procryptobia sorokini*, *Spumella* sp. and *Parabodo caudatus* served as prey. *Vitrella brassicaformis* was isolated from One Tree Island, the Great Barrier Reef from a stony coral *Leptastrea purpurea*, with *Procryptobia sorokini*, *Spumella* sp. and *Parabodo caudatus* served as prey [98]. Artificial sea water f/2 medium was used for cultivation of *V. brassicaformis* at 26°C in flat plastic tubes [98]. Solid agar plates containing 50 mg/ml kanamycin were also used to culture *V. brassicaformis* at 26°C in a 12/12 hour light/dark regime [98]. Depending on the source of *Colpodella* spp. from the environment, chromerids may also be present in the sample. In the environment, *Colpodella* spp. have been isolated from the soil, fresh water, waste water and sea water (Table 1).

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4.2. Light, differential interference contrast (DIC) microscopy and electron microscopy (EM)

For *Colpodella* species except for *Colpodella* sp. ATCC 50594, light microscopy without staining has been used to examine cells. Phase contrast microscopy, DIC, scanning electron microscopy (SEM) and transmission electron microscopy (TEM) have been used to examine the morphology and ultrastructure of cells [2, 3, 6, 83]. The use of 4',6-diamidino-2 phenylindole (DAPI) in fluorescent and confocal microscopy has been instrumental in identifying the nuclei and kinetoplasts of *P. caudatus* and of *Colpodella* sp. [7, 8]. *Colpodella* sp. ATCC 50594 has been examined with wet mounts, staining and light microscopy, DIC and TEM to examine cell morphology and ultrastructure of *Colpodella* sp. ATCC 50594 [75]. Different staining techniques that we have used to study *Colpodella* sp. ATCC 50594 have included the use of Giemsa stain, Wrights-Giemsa stain, H&E, Congo red, calcoflour, Acid-Fast, Wheatley's trichrome stain and Sam-Yellowe's trichrome staining series [8, 35, 75, 85] developed to identify and distinguish life cycle stages of *Colpodella* sp. ATCC 50594 and to distinguish cysts of *Colpodella* sp. from cysts of its prey *P. caudatus*. Sam-Yellowe's trichrome staining protocol was instrumental in aiding the identification of previously unknown developmental stages of *Colpodella* sp. ATCC 50594 and allowed the description of the life cycle of *Colpodella* sp. ATCC 50594 in culture as well as the identification of asymmetric division in the cyst stage [8]. The staining protocol is completed in less than 10 minutes. The life cycle stages of *Colpodella* sp. ATCC 50594 stained with Sam-Yellowe's trichrome stain are shown in the order of life cycle stage transitions in culture (Figure 2). We propose that this staining protocol will be highly useful in staining formalin-fixed hemolymph samples and other specimens from arthropods, blood, CSF, fecal samples and biopsied specimens for the detection and morphological characterization of *Colpodella* spp. Four staining protocols from a series that has been used in previous studies are shown in Table 3 (85, 99].

Table 3. Sam-Yellowe's trichrome staining protocols for *Colpodella* spp. staining.

Sam-Yellowe's Trichrome Staining	Application of dyes in the order of incubation
Sam-Yellowe's trichrome A	0.3 % Methylene blue (1 min) 1% Brilliant green (5 min) 1% Neutral Red (1 min) Distilled water washes were performed in between each dye incubation. After the last wash, smears are air-dried before microscope observation using oil immersion at x1000.
Sam-Yellowe's trichrome D	1% Crystal violet (30 s) 1% Brilliant green (2 min) 1% Neutral red (1 min) Distilled water washes were performed in between each dye incubation. After the last wash, smears are air-dried before microscope observation using oil immersion at x1000.
Sam-Yellowe's trichrome E	1 % Crystal violet (30 s) 1% Brilliant green (2 min) 1% Safranin (1 min) Distilled water washes were performed in between each dye incubation. After the last wash, smears are air-dried before microscope observation using oil immersion at x1000.
Sam-Yellowe's trichrome J	0.3 % Methylene blue (1 min) 0.5 % Fast green in alcohol (5 min) 1% Neutral Red (1 min) Distilled water washes were performed in between each dye incubation. After the last wash, smears are air-dried before microscope observation using oil immersion at x1000.

In our previous studies, dyes from different vendors were evaluated to establish the reproducibility and consistency of the appearance of stained cells. The staining protocol allows for the differentiation of both trophozoites and cysts of the predator and prey, and the identification of *Cryptosporidium parvum* oocysts and blood stages of *P. falciparum* [99]. Primary dyes such as methylene blue, crystal violet and Kinyoun's carbol fucshin have also been used to stain life cycle stages of *Colpodella* sp. Both dyes stain trophozoite and cyst stages but do not discriminate cyst stages

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of the predator and prey. Both *C. velia* and *V. brassicaformis* have also been studied using light microscopy, SEM and TEM [97, 98, 100], resulting in the identification of life cycle stages for both chromerids.

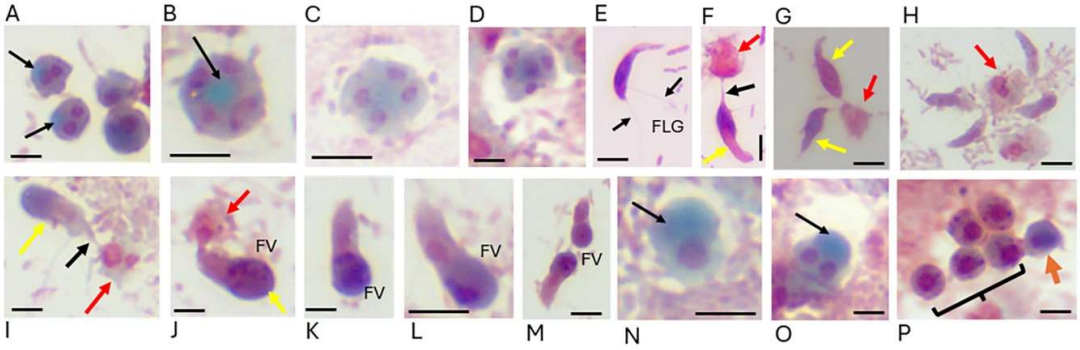

Figure 2. Sam-Yellowe's trichrome staining of *Colpodella* sp. ATCC 50594.

Life cycle stages are shown as they occur during the life cycle in culture [Getty et al.]. After preying on *P. caudatus*, *Colpodella* sp. ATCC 50594 encysts. The developmental stage of the cyst includes young demilune cysts with a single nucleus. The cyst undergoes cell division to form two or more nuclei (A-D) The black arrows shown in A and B identify the remnants of the food vacuole (FV). Excystation leads to egress of young bi-flagellated trophozoites possessing heterodynamic flagella (FLG) (E). The trophozoites attach to *P. caudatus* and feed through myzocytosis. One or more *Colpodella* sp. trophozoites can attach to a single prey during myzocytosis. Four trophozoites are shown feeding on one prey in panel H. The yellow arrows identify *Colpodella* sp., the red arrow identifies the prey *P. caudatus,* and the black arrow identifies the tubular tether that forms between predator and prey as shown in F (F-H). The trophozoite aspirates cytoplasmic contents of the prey into a posterior FV that enlarges during feeding (I & J). The black arrow in panel I is the tubular tether formed during myzocytosis. Following feeding, the unattached pre-cyst stage still containing the nucleus is shown (K-M). Flagella and cytoplasmic organelles are degraded and encystation begins (N-P). The arrows in N and O identify the remnants of the food vacuole. In panel P, the single nucleus demilune cyst of *Colpodella* sp. ATCC 50594 (orange arrow) can be distinguished from the five cysts of *P. caudatus* shown in the bracket. Scale bars: Panels A, D-K, M, O and P; 10 μm. Panels B, C, L, N; 20 μm. Cells from nutrient uptake cultures in panels A-D, and G-P were stained with Sam-Yellowe's trichrome A. Cells in panels E and F were stained with Sam-Yellowe's trichrome E. Images shown were captured at x1000 magnification as described in [7, 8]. The images shown are from archived images in the Sam-Yellowe lab. They have not been published previously.

5. *Colpodella* spp. in coinfections

Colpodella spp. detected in arthropods and vertebrate hosts have been identified coinfecting hosts with the piroplasms *Babesia* spp. and *Theileria* spp. or with *Cryptosporidium* species. Babesiosis is an emerging human tick borne zoonotic disease in different geographic regions globally [101, 102]. Human tickborne infections are considered rare events. However, the reported *Colpodella* spp. cases indicate that coinfections may be more common than expected [103]. Performing culture and microscopy can be challenging in coinfections, and PCR may not amplify target DNA in some specimens when single PCR is performed. Multiplex PCR techniques and next generation sequencing (NGS) techniques may improve detection of mixed pathogens in coinfections [103]. *Colpodella* spp. have also been detected coinfecting ticks and animal blood with bacteria such as *Anaplasma* spp., *Rickettsia* spp. and *Erlichia* spp. In coinfected hosts, ascribing the resulting pathology and tissue damage to a specific pathogen is fraught with uncertainty. The number of reports of *Colpodella* spp. infections in humans and animals has increased in the last few years (Table 4). The increased reports

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of single infections where only *Colpodella* spp. was identified and coinfections where multiple pathogens were also detected is discussed below and summarized in Table 4. Six genera of ticks; *Ixodes persulcatus*, *Rhipicephalus* (*Boophilus*) *microplus*, *Rh. bursa*, *Rh. duttoni*, *Rh. haemaphysaloides*, *Haemaphysalis longicornis*, *H. flava*, *H. bispinosa*, *H. hystricis*, *Hyalomma dromedarii*, *Dermacentor everestianus*, *D. nuttalli*, *D. andersoni*, *D. atrosignatus*, *D. taiwanensis* and *Amblyomma javanense* [10, 11, 12, 13, 14, 22, 23, 36] (Table 2) have been reported to carry *Colpodella* spp. in addition to *Stomoxys indicus* that was reported to carry *Colpodella tetrahymenae* [15].

Table 4. Increasing *Colpodella* spp. infections reported in humans and animals.

***Colpodella* spp. in humans and animals**	**Year**	**Country**	**Reference**
Human relapsing fever, non-tick associated blood infection, single infection, female	2012	China	[22]
Human relapsing fever, non-tick associated, single infection, male	2017	China	NCBI accession number MF594625
Cattle, tick associated, co-infection	2017	Mozambique	[9]
Human tickborne infection, neurological symptoms, single infection, female	2018	China	[23]
Raccoon, non-tick associated *Colpodella* spp. in the skin of the ear, co-infection	2019	Poland	[27]
Cattle, non-tick associated blood infection, co-infection	2020	Zambia	[38]
Human urinary tract infection associated with *Colpodella gonderi* and its prey *Colpoda steinii*, female	2021	Romania	[26]
Large zoo felids, *Colpodella* spp. in fecal samples, co-infection	2021	China	[17]
Dog, non-tick associated blood infection, co-infection	2021	Cambodia	[37]
Tiger (*Panthera tigris amoyensis* Hizheimer) in blood and ticks, tickborne *Colpodella* spp. infection, single infection, multiple organ damage	2022	China	[12]
Horse non-tick associated blood infection, co-infection	2022	China	[24]
Cat, non-tick associated blood infection, inflammation, tissue damage, single infection	2023	USA	[25]
Cats and dogs, non-tick associated blood infection, co-infection	2023	China	[36]
Horse, *Colpopdella* spp. in infesting biting fly (*Stomoxys indicus*), co-infection	2023	Thailand	[15]
Goats and dogs, *Colpodella* spp. in ticks	2024	China	[14]
Camels, *Colpodella* spp. in infesting ticks	2024	Egypt	[13]
Cattle and goats, *Colpodella* spp. in infesting ticks	2024	Italy	[10]
Goats and sheep, non-tick associated infection, *Colpodella* spp. in diarrhetic fecal samples, co-infection	2024	Nigeria	[18]
Pangolins, *Colpodella* spp. in infesting ticks, co-infection	2024	China	[11]
Goats, fox, duck, Eurasian Coot, non-tick associated, *Colpodella* spp. in fecal samples	2025	Cyprus	[19]

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5.1. Colpodella spp. detected in blood

Xu et al. 2022 [24] examined blood from 400 horses in China that were screened for piroplasms using primers targeting 18S rRNA gene in PCR. DNA sequences in 2 out of 400 horses showed homology to *Colpodella* spp. Phylogenetic analysis showed both identified species closely related to *Colpodella* ATCC 50594, a model isolate obtained from brown woodland soil at Gambrill State Park, Frederick, Maryland [https://www.atcc.org/products/50594 accessed 2/10/25], and to *Colpodella* sp. strains HEP (NCBI accession number GQ411073) and HLJ (NCBI accession number KT364261) isolated from two patients in China and a clade containing the apicomplexans, *Babesia* spp., *Theileria* spp., *Toxoplasma gondii*, *Cryptosporidium* spp., and *Plasmodium* spp. An additional 132 horses out of 400 were infected with *Theileria equi* and 2 out of 400 were infected with *Babesia caballi*. No symptoms were reported in the horses.

5.2. Colpodella spp. detected in blood and ticks from cattle and goats

Free grazing cattle and goats were screened for tickborne parasites. Blood and ticks from 98 cattle and 104 goats in the Puglia region, Southern Italy were screened for tickborne parasites [10]. Genomic DNA extracted from the blood and ticks were used in conventional PCR using primers targeting the 18S rRNA gene for *Babesia* spp., *Theileria* spp. and primers targeting the *gltA* gene for *Rickettsia* spp. From the cattle blood 36 out of 98 were positive for piroplasms, 7 cattle were positive for *Babesia bigemina*, 29 cattle were positive for the *Theileria sergenti/buffeli/orientalis* group, and 2 for *Sarcocystis cruzi*. From the goat blood, 39 goats out of 104 were positive for *Babesia* spp. Blood from both cattle and goats was negative for *Rickettsia* spp. Adult male and female ticks collected from cattle and goats were also examined for tick-borne pathogens. Forty-two adult male and female ticks collected from 33 cattle were evaluated. Thirty-one were identified as positive for *Rhipicephalus bursa* and eleven were positive for *Rh. seucundus*. One female *Rh. bursa* was positive for *Colpodella* spp. with 100 % sequence identity to *Colpodella* spp. with NCBI accession number OQ540588.1. In phylogenetic analysis, the identified *Colpodella* spp. DNA sequence clustered with a *Colpodella* clade from China and was close to a paraphyletic clade containing *Theileria* spp., *Babesia* spp. and *Cytauxzoon* spp. Among forty-seven adult male and female ticks collected from thirty-six goats, twenty-five were identified as *Rh. bursa*, and eleven as *Rh. secondus*. *Rickettsia* spp. were identified in 1 female *Rh. bursa.* No symptoms were reported in the cattle and goats [10].

5.3. Colpodella spp. detected in blood from dogs, cats and ticks

The presence of piroplasms in dogs and cats in Guiyang, southwestern China was investigated. *Colpodella* spp. was identified along with *Theileria* spp. Blood samples were collected from pet cats and dogs attending a veterinary hospital in Guiyang, China [36]. Genomic DNA was extracted from dog and cat blood and used for nested PCR using 18S rDNA primers targeting piroplasm parasites. Sequenced PCR products were BLAST searched and aligned with DNA sequences for *P. falciparum* and *P. berghei*, retrieved from NCBI and used for phylogenetic analysis. In pet cats *Theileria uilenbergi*, *T. luwenshuni* and *Colpodella* spp. were detected and in pet dogs *Theileria uilenbergi* and *Colpodella* spp. were detected [36]. *Colpodella* spp. from dogs and cats had 94.99% identity to *Colpodella* spp. with NCBI Accession OQ540589.1 and 81.71 % identity to each other and 81.77 % and 83 % identity to *Colpodella* sp. ATCC 50594. Cat *Colpodella* spp. with NCBI Accession OR226256 clustered in a single clade with *Colpodella* spp. from human blood with 99.60% identity but distant from gene sequences detected in ticks, horses, cattle, and tigers. Dog *Colpodella* spp. with NCBI Accession OR226258 clustered into a clade with *Colpodella* spp. NCBI accession number OQ5405891 detected in *H. longicornis* from Yiyuan County, Shandong, China with 94.99% identity but distant from *Colpodella* spp. gene sequences detected in humans, horses, cattle and tigers [36].

In another study, goats and dogs were examined in an epidemiological investigation to identify potential parasites in ticks infesting the animals in Yiyuan County in Central Shandong Province, China [14]. Genomic DNA extracted from ticks of *Haemaphysalis longicornis* was used in nested PCR

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amplification using oilgonucleotide primers targeting the 18S rRNA gene of *Theileria* and *Babesia* spp. Following amplification, a second set of primers designed to distinguish organisms in co-infections and to identify each species of *Theilaria* and *Colpodella* were also used in PCR. Coinfections of *Theileria* spp. and *Colpodella* spp. as well as individual infections with *Theileria luwenshuni* or *Colpodella* spp. were detected in ticks from goats and dogs. *Colpodella* spp. identified in goattick 168 had 93.66% identity to *Cryptosporidium stuthionis* NCBI accession number AJ697751.1 identified from farm ostriches and therefore named *Colpodella* sp. *struthionis* [14]. Goattick 161 had 92-98% identity with *Colpodella tetrahymenae* NCBI accession number MH208619.1. However, on phylogenetic analysis with sequences of *Theileria* spp., *Colpodella* spp. and *Cryptosporidium* spp. retrieved from NCBI, it was distant from other *Colpodella tetrahymenae* and *Colpodella angusta* suggestive of strain differences [14]. This species was named *Colpodella* sp. *yiyuansis* [14]. Morphological identification of the *Colpodella* species identified in *H. longicornis* is essential to determine whether the protist identified by DNA is *Cryptosporidium* or *Colpodella*. *Cryptosporidium* spp. develops oocysts which are identified in fecal samples and *Colpodella* spp. form cysts. Depending on the staining technique used, sporozoites within the oocyst of *Cryptosporidium* spp. can be discerned. *Colpodella* species develop cysts in those species that encyst. They also have trophozoite stages. Pre-cyst stages can also be identified for *Colpodella* spp. Without morphological identification of life cycle stages, errors in identification will be made leading to incorrect identifications and assigned nomenclature. No symptoms were described for the goats, dogs and cats.

In a Cambodian study, next generation sequencing (NGS) was used in investigations of dog populations where screenings were performed on blood collected from 467 dogs to identify DNA sequences of bacterial and blood borne pathogens [37]. *Colpodella* spp. along with prey protists of *Parabodo* and *Bodo* spp. were identified in one dog. The DNA sequence had 95% identity to *Colpodella* spp. identified in horse blood, NCBI accession number MW261750.1 [37]. No symptoms were reported in the dogs [37]. Other pathogens detected in the dogs screened, include the bacteria, *Mycobacterium* spp., *Coxiella* spp., *Neisseria* spp. *Rhodococcus* spp., *Treponema* spp., *Prevotella colorans*, *Mycoplasma* spp., *Rickettsia* spp. and *Wolbachia* spp. [37]. Pathogens identified from vectors such as fleas, lice, and ticks infesting the dogs include the apicomplexans, *Babesia vogeli* and *Hepatozoon canis*, the kinetoplastids *Trypanosoma evansi*, *Bodo* spp., and *P. caudatus*. The bacterial pathogens, *Anaplasma platys*, *Ehrlichia canis*, *Mycoplasma haemocanis*, *Bartonella clarridgeiae*, and *Candidatus Mycoplasma haematoparvum* were also identified [37].

5.4. Colpodella spp. detected in camel ticks, cattle and wildlife

In investigations of tick infestation in Egyptian camels in Southern Egypt, within the Luxor and Aswan governorates, 200 camels were investigated [13]. Camels were checked for ticks during regular veterinary examinations. The ticks identified belong to the species *Hyalomma dromedarii*. DNA extracted from the ticks was used as a template in nested PCR with primers targeting 18S rRNA piroplasmid genes. Thirty out of 297 ticks examined carried *Colpodella* spp. with DNA sequences homologous to *Colpodella* spp. identified in *H. longicornis* with NCBI accession number OQ540590Q, *Rh. haemaphysaloides*, NCBI accession number MH208621 and humans in China, NCBI accession number GQ411073. *Babesia bovis* was detected in 16 of the 297 ticks using the spherical body protein-4 gene. No symptoms were reported in the camels. Coinfections have also been identified in blood samples from wild animals (impalas, hartebeests, buffalos, sitatungas, lions, sable antelope and wild dogs) and cattle in the greater Kafue ecosystem in Zambia, using the V4 hypervariable region of the 18S rRNA gene of piroplasms [38]. Coinfections of *Theilaria* spp., *Babesia* spp., *Hepatozoon* spp. and *Colpodella* spp. were detected [38]. None of the animals had symptoms of infection.

5.5. Colpodella spp. identified in fecal samples from sheep, goats, cattle, duck and Eurasian Coot

Two clusters of *Colpodella* spp. were identified in phylogenetic tree analysis following detection of *Colpodella* spp. in fecal samples collected from goats and sheep in the Federal Capital Territory (FCT) and Jos, Nigeria [18]. The fecal samples were positive for *Cryptosporidium* spp. oocysts

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following microscopic examination. DNA was extracted from the fecal samples and used in nested PCR with oligonucleotide primers targeting the full length of *Cryptosporidium* spp. 18S rRNA gene. DNA from *Cryptosporidium* spp. and *Colpodella* spp. was amplified. *Colpodella* spp. was identified in 2 Red Sokoto goats, and 3 sheep of the Yankassa breed and 1 sheep of the Balami breed. Four sheep and 1 goat had diarrhea, and 1 goat had semi-formed stool [18]. DNA sequences from *Colpodella* species identified in this study had sequence identity to *Colpodella* spp. strains identified from Cyprus [19]. Two clusters consisting of *Colpodella* spp. were closely related to strains identified in a duck and fox in Cyprus, ticks infesting cattle from Mozambique [9], and a second cluster related to *Colpodella* spp. from a horse in China [18]. Fecal samples were collected from 180 animals that included goats, sheep, foxes, ducks, Eurasian Coots and Eurasian Teals in Cyprus [19]. DNA was extracted from the fecal samples and used for PCR using oligonucleotide primers targeting 18S rRNA genes from *Cryptosporidium* spp. A 600 bp DNA band identified on agarose gels and sequenced identified 1 of 10 goats, 1 of 29 foxes, 1 of 48 Eurasian Coots and 1 of 7 ducks positive for *Colpodella* spp. following a BLAST search. The four sequences were similar and had high sequence identity to *Colpodella* sp. strain HEP with NCBI accession number MH208621 from a human relapsing fever infection [22], strain HLJ with NCBI accession number KT364261 from a tickborne human infection with neurological symptoms [23] and to the *Colpodella* spp. detected in sheep from Nigeria with NCBI accession number OQ876040.1. In a Turkish study, *Colpodella* spp. was also isolated from calves with diarrhea (Table 1). The DNA sequence identified with NCBI accession number JN245625 had sequence similarity to *Colpodella* spp. DNA sequence from strain HEP identified in the human infection from Yunnan Province, China [22].

5.6. Colpodella spp. detected in pangolin ticks

Twenty-one rescued and sick Malayan pangolins infested with ticks were examined for ectoparasites at Guangdong Provincial Wildlife Rescue Center at Guangzhou Zoo and the Guangdong Institute of Applied Biological Resources in China [11]. Seventeen pangolins were found to be tick infested with *Amblyomma javanense*. Following the death of all the pangolins, DNA was extracted from tissues, blood and collected ticks for PCR. RNA was extracted for RT-PCR. Tissue sections of damaged tissues stained with H&E were also examined. No pathogens were detected in the tissues. DNA was examined for viral pathogens, bacteria and protozoan parasites. DNA sequences obtained from amplified DNA was BLAST searched, and homology obtained for *Rickettsia* spp, *Anaplasma* spp. *Erlichia* spp. *Babesia* spp., and *Colpodella* spp. were detected in ticks. *Colpodella* spp. was not detected in pangolin tissues. *Theileria* spp. and *Hepatozoon* spp. were not detected. Also, no viruses were detected. The pangolins showed symptoms of poor vigor, cough, drowsiness, anorexia, and edema of the extremities [11]. Coinfections of *Colpodella* spp. and *Rickettsia* spp., *Colpodella* spp., *Rickettsia* spp. and *Ehrlichia* spp. were detected. Bloody stools, hematuria, convulsions and other neurological symptoms were observed in the animals before death [11]. Severe organ damage, congestion and edema of major organs, ascites and inflammation were observed upon autopsy of the pangolins [11]. It is unclear what contributions *Colpodella* spp. had to the pathogenesis observed in the pangolins and whether *A. javanense* transmitted *Colpodella* spp. to the animals. Six out of 33 ticks examined carried *Colpodella* spp. Two *Colpodella* spp. had sequence identity to *Colpodella* spp. NCBI accession number MH012046 from Qinghai and *Colpodella* spp. NCBI accession number MH208621 from Yunnan. Four sequences had identity to *Colpodella* spp. NCBI accession number KT600661, the *Colpodella* species isolated from the human infection with neurological symptoms. In a study to identify the potential for the spread of pathogens by mammalian vectors in Poland, raccoons were investigated. One hundred and seventy ear fragments were collected from roadkill or from live-trapped animals in the "Warta Mouth" National Park [27]. DNA extracted from dried ear fragments was used for PCR using highly conserved primers targeting the V4 hypervariable region of the 18S rRNA gene in a semi-nested PCR. Following DNA sequencing of amplified DNA and BLAST searches, different eukaryotic organisms were identified including chlorophyta, fungi, amoeboids, uncultured colpodellidae and *Colpodella* spp. [27].

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6. *Colpodella* spp. in single infections

6.1. Colpodella spp. in human infections

A blood infection was reported in a 57 year old woman from Yunnan Province in China with an immunodeficiency of natural killer cells [22]. Symptoms of productive cough, malaise, hemolytic anemia and relapsing illness was reported [22]. Oligonucleotide primers targeting a conserved fragment of *Babesia* spp. 18S rRNA was used in PCR and the gene amplified matched with 89% identity to *Colpodella tetrahymenae* [22]. Following treatment with atovaquone and azithromycin, the *Colpodella* infection was cleared [22]. Anti-*Colpodella* antibodies from the patient reacted with *Colpodella* in erythrocytes by IFA. In a second case of relapsing fever reported in a male patient, *Colpodella* DNA sequences with NCBI accession number MF594625 were also identified. However no further details were provided for the patient. A 55 year old female patient with neurological symptoms following a tick-bite, from Heilongjiang Province of Northeast China was also reported [23]. Her symptoms included fever, dizziness, gait disturbance, and headache [23]. Polymerase chain reaction of blood and CSF using primers targeting *Babesia* spp. identified *Colpodella* spp. in CSF but not in the blood sample [23]. Treatment with doxycycline cleared the infection. In both human cases, *Babesia* spp., *Eperythrozoon* spp. [Yuan et al.], *Borellia* spp., *Borellia burgdoferi* sensu lato (sl), *Babesia* spp., tick-borne encephalitis virus (TBEV), *Anaplasma* spp., *Ehrlichia* spp. and *Rickettsia* spp. were negative by PCR [23]. Although an IgG titer of 1:126 in the serum of the patient was obtained for *B. burgdoferi* leading to suspected Lyme disease, PCR was negative [23]. Two *Colpodella* spp. were detected in 2 out of 474 adult *Ixodes persulcatus* from woodlands around the patients' home [23]. Both DNA sequences with NCBI accession numbers KT600661 and KT60062 shared 93.8% identity but had 88.0-89.0% identity to the tick *Colpodella* sp. strain HLJ from the patient [Jiang et al.]. *Colpodella gonderii* along with its prey *Colpoda steinii* was identified in the urine of a 70 year old female patient with a history of chronic diseases, in Cluj-Napoca, Romania by wet-mount microscopy and Giemsa staining [26]. No other parasites were detected. The patient was admitted for respiratory symptoms including breathing difficulties, dyspnea with orthopnea but no urinary symptoms [26]. Giemsa staining identified *Colpodella gonderii* and *Colpoda steinii* trophozoites. After treatment with ceftriaxone and metronidazole, both protists could no longer be detected in the urine [26]. Standard symptoms for colpodellosis have not been described. However, in the relapsing infection [22], *Babesia*-like symptoms of anemia, elevated reticulocyte and lactate dehydrogenase levels were reported [22].

6.2. Colpodella spp. detected in fecal samples from large cats

Fifty-six fresh fecal samples from a Siberian tiger, White tiger, Bengal tiger, African tiger, White lion, Lynx, and Jaguar from Harbin Zoo, China were collected to screen for *Cryptosporidium* spp. [17]. DNA was extracted from the fecal samples and amplified with PCR using oligonucleotide primers targeting 18S rDNA sequences of *Cryptosporidium* sp. In 7 out of 56 samples *Colpodella* spp. DNA was amplified and showed a band of 583 bp on agarose gels. DNA sequence identity of the 18S rRNA gene was 97.26% between *Cryptosporidium* and *Colpodella* spp. DNA sequences. No symptoms were reported in the large cats [17].

Twenty-two *Colpodella* species were detected in ticks (3 genera, 8 species) from the Meihua Mountains, China using nested PCR with universal primers targeting 18S rRNA gene and template DNA extracted from the blood of a South China tiger and DNA extracted from ticks [12]. Four hundred and two ticks were examined of which 22 contained *Colpodella* spp. Among the ticks, *Dermacentor andersoni* contained 2 *Colpodella* sp.; *D. atrosignatus* contained 1 *Colpodella* sp.; *D. taiwanensis* contained 1 *Colpodella* sp.; *Haemaphysalis longicornis* contained 3 *Colpodella* sp., (*Colpodella* sp. strain HLJ); *H. hystricis*, contained 3 *Colpodella* sp., (1 *Colpodella* sp. strain HLJ); *H. bispinosa*, contained 1 *Colpodella* sp., (1 *Colpodella* sp. strain HLJ); *H. flava*, contained 2 *Colpodella* sp., 3 *Colpodella* sp. strain HEP; and *R. duttoni*, contained 1 *Colpodella* spp. [Chiu et al.]. The PCR primers used for nested PCR that amplified *Colpodella* spp. 18S rRNA gene were designed to target the 18S rRNA gene from piroplasms as described by Matsimbe et al. [9]. The study by Chiu et al. [12] highlights an

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important factor in the investigations to understand the disease characteristics of *Colpodella* spp. The aim of the study was not to identify *Colpodella* species but to identify tickborne pathogens like piroplasms, with the potential for zoonotic transmission of tickborne pathogens, due to the close contact between humans and wild life in the region investigated. The PCR was positive for *Colpodella* with 22 *Colpodella* spp. identified. The PCR was negative for *Mycoplasma suis* and *T. gondii*. In suspected cases of piroplasm infection, the presence of *Colpodella* spp. should also be determined as a possible coinfecting species and possibly the only infecting species. Similarly, in suspected cases of cryptosporidiosis, *Colpodella* infection should also be suspected. DNA sequences from the blood of the tiger and DNA sequences from the ticks had 100% similarity. Both had 90.1 % sequence identity to *Colpodella* sp. HEP (human erythrocyte parasite) from *Colpodella* sp. infecting human red blood cells and 90.4 % identity to *Colpodella* sp. strain HLJ (Heilongjiang) from the human patient with neurological symptoms. Based on DNA analysis, Chiu et al. [12] propose that the tiger became infected through the bite of *Haemaphysalis flava* identified morphologically and by 16S and ITS2 rRNA sequences. Strains HEP and HLJ have been reported in human infections [22, 23]. *Colpodella* spp. was also detected in water samples from ditches around the tiger enclosure but not in soil samples from the same area [12]. Symptoms in the tiger after tick bite included anorexia, runny nose, drool, and bluish-green stool [12]. The tiger was treated with sulfonamide, cephalosporin, and ampicillin. Pathology following death of the tiger included whole-body severe jaundice, including skin, eye conjunctiva, oral mucosa, trachea and coronary fats around the heart. Hepatomegaly, splenomegaly, hemorrhage in the kidney and mesenteric lymph nodes was also observed [12]. The authors propose that the ticks might have sucked *Colpodella* sp. along with water from the ditches and transmitted *Colpodella* to the tiger during the tick bite [12]. Sixteen of the 22 ticks carrying *Colpodella* spp. were of the genus *Haemaphysalis*. The species *Haemaphysalis flava* and *H. longicornis* were the dominant ticks identified. *H. flava* also transmits *Babesia* spp. *Colpodella* spp. was the only pathogen identified in the ticks. Even among the same *Colpodella* strains, phylogenetic analysis showed that sequences clustered in separate groups. There is an urgent need to define *Colpodella* species and strain relationships, morphologically, serologically, molecularly and biochemically to better understand strain distributions globally, transmission patterns and the epidemiology of colpodellosis in the different geographic regions.

7. Conclusions and Recommendations

The detection of *Colpodella* species in arthropods, humans and animals continues to increase. Infections appear to occur by both direct and tickborne routes, with symptoms and pathogenesis reported through both routes. It is unclear if the direct transmission may also be tickborne and if these infections are opportunistic or zoonotic. The increasing number of reports of *Colpodella* spp. detection in environments associated with human activity and close to human dwellings poses a public health concern due to the vulnerability and susceptibility of immunocompromised or chronically ill individuals that can become infected by *Colpodella* spp. It is unclear if the human infections are age or gender biased since there have only been four cases reported, with three women and one man. Due to the frequent reports of *Colpodella* spp. in ticks biting humans and animals, and known to transmit zoonotic bacterial and protozooan infections, *Colpodella* spp. is also suspected to be a zoonoses. However evidence is currently lacking to confirm zoonotic infections. However, as investigations continue in this area, transmission patterns will emerge. The most pressing issue facing the understanding of colpodellosis and *Colpodella* spp. as a pathogen, is that the life cycle stages causing disease transmission and pathogenesis are unknown. The morphology of *Colpodella* spp. has not been described in any of the tick associated infections, in any of the animals that had *Colpodella* species in blood samples and in any of the fecal samples examined. *Colpodella gonderi* was identified in the urine of a chronically ill elderly woman by Giemsa stain. Characteristic identifiable symptoms of colpodellosis in humans and animals that can be distinguished in cases of coinfections with bacteria, piroplasms or *Cryptosporidium* spp. are unknown. While the use of PCR to amplify the 18S rRNA gene is useful and has demonstrated the close phylogenetic relationship of *Colpodella* species

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with the piroplasms and *Cryptosporidium* spp., the morphology of the different *Colpodella* species and strains are still unknown and molecules participating in the mechanisms of transmission, nutrient uptake and survivial within the arthropod, human and animal hosts, and mechanisms of pathogenesis are unknown. These must be investigated to understand the biochemistry, cell and molecular biology of *Colpodella* spp. Kelch 13 and coronin genes were recently detected in *Colpodella* sp. ATCC 50594 as markers of endocytosis. Additional gene discovery investigations are needed to understand the role of encoded proteins in disease transmission and pathogenesis. When *Colpodella* spp. are identified in arthropods, and in human and animal specimens, the specimens should be stained for microscopy to identify life cycle stages of *Colpodella* spp. Staining protocols have been developed that can distinguish *Colpodella* spp. life cycle stages from those of prey that may be present in specimens. Blood should be collected from humans and animals infected for obtaining antiserum to be used for evaluation of antibodies specific to *Colpodella* spp. antigens. Culture of specimens containing *Colpodella* spp. will provide cell pellets that can be extracted for protein, DNA or RNA and for immunological and molecular biology investigations. Primers targeting the 18S rRNA genes of the bodonid, algae and ciliate prey species should also be used for PCR amplification to determine the presence of the prey species in arthropods and host samples. Specimens containing *Colpodella* spp. should be cultured to propagate the identified species. The prey species for *Colpodella* spp. may be present in the specimen and can be cultured along with *Colpodella* spp. Media can be bacterized before use and *Bodo* spp. and *Parabodo* spp. can be obtained in monoprotist cultures from the ATCC and added to the culture to maintain growth of *Colpodella* spp. When epidemiological screenings are performed for tickborne bacteria, piroplasms or for *Cryptosporidium*, the presence of *Colpodella* spp. should be suspected and screened. Prolonged symptoms non-responsive to conventional treatments following tick bite or biting flies with unknown etiology in humans or animals should be evaluated for *Colpodella* spp. Similarly, symptoms of diarrhea in suspected cases of cryptosporidiosis should be evaluated for the presence of *Colpodella* spp. Proper prevention, management, diagnosis and treatment of colpodellosis will require an integrated approach that includes staining and microscopy, morphological charaterization, nucleic acid amplification and immunoassays.

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