Sunil Thomas *Editor*

Rickettsiales

Biology, Molecular Biology, Epidemiology, and Vaccine Development

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Dedicated to all the
Infectious Disease
Researchers and Medical Practitioners

Preface

Infectious diseases are the leading cause of death in children and adolescents. Development of effective vaccines to protect against infectious diseases not only helps in the well-being of humans but also decreases mortality in farm animals and fishes, thus increasing food security. Vaccination programs are also effective in reducing the reliance on antibiotics, the abuse of which has led to antibiotic resistant microorganisms as well as destruction of the beneficial microbiome leading to several metabolic diseases. As yet vaccination has eradicated only two diseases—small pox in humans and rinderpest in cattle. Aggressive vaccination strategies have lowered the incidence of many diseases including polio, rubella, and Guinea worm disease. However, currently, there are no commercially available vaccines for none of the diseases caused by members of the order *Rickettsiales*.

Many members of the order *Rickettsiales* are emerging and re-emerging diseases and are transmitted through insect bites or by consuming contaminated fish with infected trematodes. Diseases caused by *Rickettsiales* are often misdiagnosed leading to delayed treatment resulting in fatal outcomes. Changes in climate have opened up new ranges for the insect vectors, and hence the incidence of the disease caused by *Rickettsiales* (genus *Anaplasma, Ehrlichia, Orientia, Rickettsia,* and *Neorickettsia)* has increased in humans and animals over the years.

There are very few published books on the order *Rickettsiales*. This book *Rickettsiales: Biology, Molecular Biology, Epidemiology, and Vaccine Development* is intended for students, researchers, and professors interested in members of the order *Rickettsiales*, their biology, mode of infection, immunology, and development of vaccines. The book has an introductory chapter on the importance, diagnosis, treatment, and management of *Rickettsiales* diseases. There are also sections on *Ehrlichia*, *Anaplasma, Rickettsia, Orientia, Neorickettsia*, *Wolbachia*, and *Midichloraceae*. Knowledge on the physiology of *Wolbachia* has led to the use of the bacteria in integrated pest management strategies. As *Wolbachia* is known to curb the ability of mosquitoes to transmit Zika virus, they are now employed to prevent the transmission of these viruses.

My sincere thanks to all the authors who contributed chapters to the book in spite of their busy schedule. This book would not have materialized without the insightful knowledge, substantial effort as well as timely contributions by the authors. Finally, my heartfelt thanks to my wife Jyothi and children Teresa and Thomas for the encouragement and support to complete the book in a very short time.

Wynnewood, PA Sunil Thomas

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Part I
Rickettsiales: Importance and Taxonomy

Chapter 1
The Importance of *Rickettsiales* Infections

Sunil Thomas, Walker Alexander, John Gilligan, and Yasuko Rikihisa

1 Introduction

Bacteria of the order *Rickettsiales* are obligate intracellular parasites that infect a variety of hosts. The order *Rickettsiales* comprises the families: (1) *Rickettsiaceae*, with the genera *Rickettsia*, *Orientia*, *Occidentia*, "*Candidatus* (*Ca.*) Megaira", "*Ca.* Cryptoprodotis", "*Ca.* Arcanobacter", "*Ca.* Trichorickettsia", and "*Ca.* Gigarickettsia"; (2) *Anaplasmataceae*, with the genera *Anaplasma*, *Wolbachia*, *Ehrlichia*, *Neorickettsia*, *Aegyptianella*, "*Ca.* Neoehrlichia", "*Ca.* Xenohaliotis", and "*Ca.* Xenolissoclinum"; and (3) *Midichloriaceae* (Montagna et al. 2013; Szokoli et al. 2016a, b). All bacteria in the order *Rickettsiales* are Gram-negative Alphaproteobacteria. The *Rickettsiales* are widely regarded as being the closest relatives to mitochondria.

The common features of *Rickettsia* are their intracellular growth environment, their small genome sizes (1.1 Mb), and their low genomic G + C contents (29–33 %). The genus *Rickettsia* has an unusual arrangement of the rRNA genes. The 5S, 16S, and 23S rRNA genes are linked together in other bacteria, whereas in members of *Rickettsia* the 16S rRNA gene is separated from the 23S and 5S rRNA gene cluster and the 23S rRNA gene is preceded by a gene which codes for methionyl-tRNAf(Met) formyltransferase (Andersson et al. 1999).

The bite or inoculation of infectious fluids or feces from the ectoparasites such as fleas, lice, mites, and ticks are involved in the transmission of the pathogenic *Rickettsiales* including *Rickettsia*, *Orientia*, *Ehrlichia*, and *Anaplasma.* However, *Neorickettsia* are transmitted by ingestion of endoparasites (trematodes). *Neorickettsia*

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are vertically transmitted endosymbionts of parasitic flukes (Phylum: Platyhelminthes; Class: Trematoda; Subclass Digenea). Sennetsu fever, caused by *Neorickettsia sennetsu* is contracted by eating raw fish that are the vertebrate hosts of the flukes (Rikihisa et al. 2004; Dittrich et al. 2015); whereas, *Neorickettsia risticii* causes the Potomac horse fever. Notable exception in the order *Rickettsiales* is the *Wolbachia* that are maternally inherited intracellular bacterial symbionts that infect more than 60 % of all insect species (Moreira et al. 2009) and nematode (Taylor et al. 2005; Werren et al. 2008). Members of the family *Midichloriaceae* are also symbionts infecting insects, fish, and animals. *Candidatus* Midichloria mitochondrii is a bacterium that resides within animal mitochondria. They are localized both in the cytoplasm and in the intermembrane space of the mitochondria of ovarian cells of ticks (Sassera et al. 2006).

All age groups of humans and animals are at risk of infections by *Rickettsiales* especially in endemic areas. Both short- and long-term travelers are at risk for infection. Transmission is increased during outdoor activities in the spring and summer months when ticks, fleas, trematode-infected fish, and insects are most active. However, infection can occur throughout the year. Because of the 5- to 14-day incubation period for most rickettsial diseases, tourists may not necessarily experience symptoms during their trip, and onset may coincide with their return home or develop within a week after returning. Although the most commonly diagnosed rickettsial diseases in travelers are usually in the spotted fever or typhus groups, travelers may acquire a wide range of rickettsioses, including emerging and newly recognized species (Source: Centers for Disease Control and Prevention [CDC]).

2 Disease Caused by *Rickettsia*

The *Rickettsiaceae* are a family of obligate intracellular small Gram-negative coccobacilli which infect humans chiefly through insect vectors, mostly from animal hosts, but sometimes by transovarial transmission in the insects themselves (Cowan 2000). *Rickettsia* is the most prominent member of the family *Rickettsiaceae.* Rickettsioses are caused by obligate intracellular bacteria and include the typhus group and the spotted fever group of the genus *Rickettsia*. These zoonoses are among the oldest known vector-borne diseases (Parola et al. 2013). Currently, 29 *Rickettsia* species with validated and published names have been reported (http://www.bacterio.cict.fr/qr/rickettsia.html).

The most important species of *Rickettsia* includes:

R. prowazekii is the agent of epidemic typhus, transmitted by the human body louse (clothing), *Pediculus humanus* (but not by head lice) from active human cases or from healthy carriers or subclinical cases (Brill-Zinsser disease). The infectious agent in the feces of the body louse is usually inoculated by scratching of the site of the louse bite. Inhalation of an aerosol of dried louse feces in closed communities is sufficient to cause epidemics (Cowan 2000).

R. typhi (murine typhus) is the causal agent of endemic typhus, is carried by the rat flea *Xenopsylla cheopis*, and typically infects man in markets, grain stores, breweries, and garbage depots (Cowan 2000). It is often a mild illness, but can become more aggressive in refugee camps. Endemic typhus is highly treatable with antibiotics. Most people recover fully, but death may occur in the elderly, severely disabled or patients with a depressed immune system. The most effective antibiotics include tetracycline and chloramphenicol.

R. rickettsii is the agent of Rocky Mountain spotted fever. The pathogen is transmitted to humans by the bite of infected tick species. In the United States, these include the American dog tick (*Dermacentor variabilis*), Rocky Mountain wood tick (*Dermacentor andersoni*), and brown dog tick (*Rhipicephalus sanguineus*). Typical symptoms include fever, headache, abdominal pain, vomiting, and muscle pain. A rash may also develop, but is often absent in the first few days, and in some patients, never develops. Rocky Mountain spotted fever can be a severe or even fatal illness if not treated in the first few days of symptoms. Doxycycline is the first-line treatment for adults and children of all ages, and is most effective if started before the fifth day of symptoms (Source: CDC).

R. conorii is responsible for the disease Boutonneuse fever, Mediterranean spotted fever, Israeli tick typhus, Astrakhan spotted fever, Kenya tick typhus, Indian tick typhus, or other names that designate the locality of occurrence while having distinct clinical features. The pathogen is transmitted by the brown dog tick *Rhipicephalus sanguineus*.

R. africae is the causative agent of African tick-bite fever, is transmitted by *Amblyomma hebraeum* and *A. variegatum* ticks. African tick-bite fever is a neglected disease that has been mainly detected in tourists who are bitten by ticks while traveling in disease-endemic areas.

R. akari is the causative agent of rickettsialpox. Rickettsialpox is a cosmopolitan, mite-borne, spotted fever rickettsiosis. The disease is characterized by a primary eschar, fever, and a papulovesicular rash. Rickettsialpox was first identified in New York City in 1946 (Paddock et al. 2006). The arthropod vector of *R. akari* includes the house mouse mite (*Liponyssoides sanguineus*), and the principal rodent host, the house mouse (*Mus musculus*), brown rat (*Rattus norvegicus*), and reed vole (*Microtus fortis pelliceus*).

3 Disease Caused by *Orientia*

Orientia tsutsugamushi is the causative organism of scrub typhus. Scrub typhus is transmitted by infected mites [(trombiculid mite larvae) (*Leptotrombidium* spp.), commonly called chiggers] encountered in high grass and brush, is endemic in northeastern Japan, southeast Asia, the western Pacific Islands, eastern Australia, China, and parts of south-central Russia, India, and Sri Lanka. An estimated one billion people in the endemic area are at risk for scrub typhus and an estimated one million new cases occur annually. Most travel-acquired cases of scrub typhus occur during

visits to rural areas in endemic countries for activities such as camping, hiking, or rafting, but urban cases have also been described. Scrub typhus is an acute febrile illness characterized by fever, rash, and eschar, and often leads to severe clinical complications such as interstitial pneumonia, acute renal failure, meningoencephalitis, gastrointestinal bleeding, and multi-organ failure. Mortality rates for scrub typhus range from <1 to 50 % depending upon proper antibiotic treatment, health status of the patient, and virulence of the infected strain of *O. tsutsugamushi* encountered. Scrub typhus can be effectively treated with antibiotics including doxycycline, chloramphenicol, and azithromycin. However, re-infection is common due to the wide variety of antigenically distinct serotypes (Min et al. 2014). As yet there are no vaccines to protect against *Orientia* (Kelly et al. 2009).

4 Disease Caused by *Ehrlichia*

Ehrlichiosis is the general name used to describe several bacterial diseases that affect animals and humans. Human ehrlichiosis is a disease caused by at least three different ehrlichial species in the United States: *Ehrlichia chaffeensis*, *Ehrlichia ewingii,* and *Ehrlichia muris*-like (EML). Ehrlichiae are transmitted to humans by the bite of an infected tick. The lone star tick (*Amblyomma americanum*) is the primary vector of both *Ehrlichia chaffeensis* and *Ehrlichia ewingii* in the United States. Typical symptoms include fever, headache, fatigue, and muscle aches. Usually, these symptoms occur within 1–2 weeks following a tick bite. Ehrlichiosis is diagnosed based on symptoms, clinical presentation, and later confirmed with specialized laboratory tests. The first-line treatment for adults and children of all ages is doxycycline (Source: CDC; Rikihisa 1991; Paddock and Childs 2003; Rikihisa 2010).

5 Disease Caused by *Anaplasma*

Anaplasmosis is a tick-borne disease caused by the bacterium *Anaplasma phagocytophilum.* It was previously known as human granulocytic ehrlichiosis (HGE) and has recently been renamed as human granulocytic anaplasmosis (HGA). Anaplasmosis is transmitted to humans by tick bites primarily from the black-legged tick (*Ixodes scapularis*) and the western black-legged tick (*Ixodes pacificus*). Of the four distinct phases in the tick life-cycle (egg, larvae, nymph, adult), nymphal and adult ticks are most frequently associated with transmission of anaplasmosis to humans. The mammalian reservoir for *A. phagocytophilum* infection within the United States includes white-footed mice (*Peromyscus leucopus*), raccoons (*Procyon lotor*), gray squirrels (*Sciurus carolinensis*), gray foxes (*Urocyon cinereoargenteus*), and redwood chipmunks. Typical symptoms of the disease include fever, headache, chills, muscle aches, hematological abnormalities, including leucopenia and thrombocytopenia; and increased serum aminotransferase liver enzyme

activity, which suggests mild to moderate liver injury. Usually, these symptoms occur within 1–2 weeks of a tick bite. Anaplasmosis is initially diagnosed based on symptoms and clinical presentation, and later confirmed by the use of specialized laboratory tests. The first-line treatment for adults and children of all ages is doxycycline (Rikihisa 2003, 2010, 2011).

6 Disease Caused by *Neorickettsia*

Neorickettsia sp. are found in various species of trematodes (flukes) (endoparasites of vertebrates and invertebrates). When humans ingest metacercaria stage of infected trematodes encysting in the fish, *Neorickettsia sennetsu* is transmitted to humans causing sennetsu fever (Rikihisa et al. 2005; Dittrich et al. 2015). Whereas, when dogs ingest the metacercaria stage of infected trematodes encysting in the fish, *Neorickettsia helminthoeca* is transmitted to dogs causing salmon poisoning disease (Headley et al. 2011). When horses ingest metacercaria stage of infected trematodes encysting in the aquatic insects (mayflies, caddisflies), *Neorickettsia risticii* is transmitted to horses and cause Potomac horse fever. The first line of treatment is the antibiotic doxycycline (humans and dogs) and oxytetracycline (horses) (Gibson et al. 2005; Rikihisa 2006; Lin et al. 2009).

7 Significance of *Wolbachia*

Wolbachia are intracellular bacteria that are found in arthropods and nematodes. *Wolbachia* is the most renowned insect symbiont, due to its ability to manipulate insect reproduction and to interfere with major human pathogens thus providing new avenues for pest control (Bourtzis et al. 2014). These alphaproteobacteria endosymbionts are transmitted vertically through host eggs and alter host biology in diverse ways, including the induction of reproductive manipulations, such as feminization, parthenogenesis, male killing, and sperm–egg incompatibility. They can also move horizontally across species boundaries, resulting in a widespread and global distribution in diverse invertebrate hosts (Werren et al. 2008). Mosquitoes are responsible for the transmission of arboviruses including Dengue, Chikungunya, and Zika viruses. Recent studies demonstrate that *Wolbachia pipientis* could be used to control mosquito (*Aedes aegypti*) population (Iturbe-Ormaetxe et al. 2011), thereby controlling dengue and chikungunya infection (Moreira et al. 2009; Hoffmann et al. 2011; Walker et al. 2011; Bull and Turelli 2013; Aliota et al. 2016). *Aedes aegypti*-harboring *Wolbachia* are highly resistant to infection with two strains of Zika virus. *Wolbachia*-harboring mosquitoes displayed lower viral prevalence and intensity and decreased disseminated infection and, critically, did not carry infectious virus in the saliva, suggesting that viral transmission was blocked (Dutra et al. 2016).

Commercial enterprises have come up with several strategies to control Zika virus. One of the strategies involves rearing mosquitoes infected with a particular strain of *Wolbachia* and releasing the males into the environment. When these male mosquitoes mate with wild females that do not carry the same strain of *Wolbachia*, the resulting fertilized eggs do not hatch as the paternal chromosomes are not properly developed. As infected male mosquitoes continue to be released to breed with wild partners, the pest population dwindles (Waltz 2016). It is clearly not understood how *Wolbachia* inhibits viruses in mosquitoes; though it is thought that *Wolbachia* produce a cellular lipid environment that is antagonistic to viral replication (Molloy et al. 2016).

Though *Wolbachia* causes negative effects in arthropods, a *Wolbachia* strain associated with the bedbug *Cimex lectularius*, designated as *w*Cle, was shown to be essential for normal growth and reproduction of the blood-sucking insect host via provisioning of B vitamins (Hosokawa et al. 2010). *Wolbachia* has also been described from nematode hosts such as those responsible for onchocerciasis (river blindness), lymphatic filariasis (elephantiasis), and dirofilariasis (heartworm), where the symbiotic interaction has features of mutualism. Wolbachial symbiont is essential for the life of some of nematode species, as such antibiotic against *Wolbachia* can be used as anti-filarial drug treatment (Taylor et al. 2013).

8 Prevention of *Rickettsiales* Diseases

As yet there are no vaccines for any diseases caused by the bacteria of the order *Rickettsiales*. Antibiotics are not recommended for prophylaxis of rickettsial diseases. Travelers are instructed to minimize exposure to infectious arthropods during travel (including lice, fleas, ticks, mites) and animal reservoirs (particularly dogs) when traveling in endemic areas. The proper use of insect or tick repellents, self-examination after visits to vector-infested areas, and wearing protective clothing are ways to reduce risk (Source: CDC).

9 Taxonomy

The order *Rickettsiales* are obligatory intracellular Gram-negative bacteria belong to the group alphaproteobacteria. The order *Rickettsiales* consists of the family *Rickettsiaceae* and the family *Anaplasmataceae*. The family *Rickettsiaceae* contains the genus *Rickettsia* and the genus *Orientia*. The family *Rickettsiaceae* are short rods or coccobacilli but the family *Anaplasmataceae* are small pleomorphic cocci. The rickettsiae-causing diseases in humans belong to the families *Rickettsiaceae* and *Anaplasmataceae*. The family *Rickettsiaceae* include two genera. The genus *Rickettsia* comprises the highly related typhus group (TG)

and spotted fever group (SFG) and the genetically heterogeneous species *Orientia tsutsugamushi* that includes several serovars. The family *Anaplasmataceae* is currently comprised of seven established genera and *Candidatus* genera: *Ehrlichia, Anaplasma, Aegyptianella, Neorickettsia, Wolbachia*, "*Candidatus* Neoehrlichia", and "*Candidatus* Xenohaliotis".

10 Epidemiology

The rickettsiae are endemic worldwide. Rickettsiae are known or thought to be associated with invertebrates (arthropods and trematodes). The invertebrates are also the vectors that transmit the rickettsiae in human and other vertebrates. Humans are accidental hosts to the rickettsiae, except for epidemic typhus and recrudescent typhus or Brill-Zinsser disease. Some species of the rickettsiae within a biogroup appear to be confined to certain geographic areas of the world. These species acquire some biologic, pathogenic, and genetic characteristics within their respective area that make them different enough from each other within a biogroup to be classified as a new species, e.g., the SFG *Rickettsia japonica* is confined to Japan, and *Rickettsia rickettsii* appears to be limited to the Western Hemisphere. *Rickettsia felis*, a newly isolated *Rickettsia* that causes an endemic typhus-like syndrome, has at present only been isolated in the Western hemisphere. *Rickettsia conorii* is primarily found in the Mediterranean basin and Africa, and *O. tsutsugamushi* is found in Southeast Asia and Japan. In contrast, other species are ubiquitously found in various geographic areas of the world and appear to be biologically, genetically, and pathologically similar or nearly identical. Examples are *Rickettsia typhi*, which causes endemic typhus. Epidemic typhus caused by *Rickettsia prowazekii* was worldwide in the past. However, at present it appears to be confined to foci in areas of Africa, areas of the former Soviet Union, and South America. Sennetsu fever caused by *N. sennetsu*, which was thought to be limited to Asia and Japan, has now been reported in other geographic regions. In the United States, human monocytic ehrlichiosis (HME) is caused by *Ehrlichia chaffeensis*. In addition, a granulocytic anaplasmosis, which is caused by *A. phagocytophilum,* and was initially reported in equines, ruminants, and canines, is now found in humans (HGA). *Ehrlichia ewingii,* once thought to infect only dogs, has recently discovered to cause human granulocytic ehrlichiosis (Human ewingii ehrlichiosis). In the United States, RMSF, both types of endemic typhus caused by *R. typhi* or *R. felis*, HME and HGA are the most prevalent diseases. Most cases occur between May and September. Between May and September, environmental conditions are optimal for tick activity and human outdoor activities peak. However, a few cases have also been diagnosed during the winter months. Therefore, rickettsial diseases cannot be considered strictly seasonal. In the United States, most of the RMSF cases are east of the Rockies, with Oklahoma and the Carolinas leading the states. Ninety percent of endemic typhus cases in the United States are detected in the Southwest, especially in southern Texas, and in the West in southern California. A

few endemic typhus cases have been reported from Virginia, North Carolina, Oklahoma, and California. Classic louse borne epidemic typhus is not known to occur in the United States. However, a rickettsial organism closely resembling *R. prowazekii* was isolated from a flying squirrel (Hechemy et al. 2006).

11 Pathobiology

The intracellular sites of growth are different in members of the order *Rickettsiales*. Members of the SFG rickettsiae, e.g., *R. rickettsii*, grow in the cytoplasm and sometimes in the nucleus. In contrast, members of the TG, e.g., *R. typhi*, and the scrub typhus grow in the cytoplasm. Members of the family *Anaplasmataceae* replicate in the membrane-bound compartment that does not fuse with lysosomes. The replication site for *E. chaffeensis* within the cell is the early endosome, and those of *A. phagocytophilum* is early autophagosomes (Rikihisa 2011, 2015).

Except for *Wolbachia* and "*Candidatus* Xenohaliotis", members of the family *Anaplasmataceae* infect cells of hematopoietic and bone marrow origin of mammals or birds. Generally wild animals are reservoirs of these bacterial infection and humans and domestic mammals or birds are infected by the bite of ticks infected with *Ehrlichia* or *Anaplasma* sp., or by ingestion of trematodes infected with *Neorickettsia*. *Wolbachia* is so far known to infect only invertebrate cells. However, it can be found in the blood stream of humans when released from filarial worms infesting the vertebrates. Among *Ehrlichia* species, so far *E. chaffeensis*, *E. ewingii*, and *E. canis* have been isolated and/or detected in the blood specimens from humans. In the genus *Anaplasma*, *A. phagocytophilum* and *A. platys*, and in the genus *Neorickettsia*, *N. sennetsu* are the species so far documented in humans.

No exotoxin has been reported to explain the pathogenic properties of the *Rickettsiaceae* and *Anaplasmataceae*. The diseases caused by the rickettsias are systemic illnesses exhibiting protean manifestations. The hallmark of the various diseases caused by the SFG and TG rickettsiae is the maculopapular rash; however, it is not found in every case. In SFG rickettsiae, it begins on the wrists and ankles and extends throughout the body. In scrub typhus and Mediterranean spotted fever (MSF), an eschar may develop at the site of the insect bite. In TG rickettsiae, the rash is usually centrally distributed on the trunk and rarely involves the palms and soles. The internal lesions caused by the pathogen are a vasculitis localized in the endothelium and smooth muscle. Vascular permeability is increased, causing various degrees of hemorrhage, tissue edema, and peripheral circulatory failure. The extent of the internal vascular lesions is related to the degree of pathogenicity of a given species, e.g., in scrub typhus, vascular damage is not usually as severe as that seen in RMSF (Hechemy et al. 2006).

Human ehrlichiosis and anaplasmosis also exhibits nonspecific protean manifestations (Rikihisa 2010, 2015), which are similar to those observed in patients

with RMSF. However, most patients do not have a rash. The onset of illness is abrupt. Symptoms include fever, chill, headache, myalgia, anorexia, nausea or vomiting, and weight loss. Thrombocytopenia, leucopenia, and liver enzyme abnormality are often reported. A meningitis syndrome or an encephalitis or encephalopathy syndrome may occur with HME, and *E. chaffeensis* may be detected in the cerebrospinal fluid. Fatal seronegative infection has been reported in HIV patients, and secondary infections due to *Anaplasma*-induced immunosuppression may lead to severe diseases and death. The patients also lack remarkable lesions such as cell lysis, tissue necrosis, abscess formation, or severe inflammatory reactions. The monocyte and granulocyte are the primary target cells for monocytic ehrlichiosis and granulocytic anaplasmosis/ehrlichiosis, respectively.

12 Vectors of *Rickettsiales*

12.1 Tick Vectors

The bacteria of the order *Rickettsiales* are usually spread to people through the bites of ticks, mites, fleas, or lice that previously fed on an infected animal. Organisms can be transmitted by bites from these ectoparasites or by the inoculation of infectious fluids or feces from the ectoparasites into the skin. Inhaling or inoculating conjunctiva with infectious material may also cause infection for some of these organisms. Tick-transmitted rickettsial diseases, such as ehrlichiosis and spotted fever rickettsiosis, are significant sources of morbidity and mortality (Source: CDC).

Ticks are members of the phylum Arthropoda, subphylum Chelicerata, class Arachnida, and subclass Acari (Keirans and Durden 2005). The tick family includes: *Ixodidae* (hard ticks), and *Argasidae* (soft ticks). These two groups differ in their anatomy, behavior, and life cycle (Bogitsh et al. 2005). The Ixodidae are called hard ticks because of their hard dorsal shield (scutum) and they attach to their host for prolonged periods. The hard ticks (Figs. 1.1 and 1.2) exhibit sexual dimorphism and are more commonly encountered by animals and people than soft ticks. The Argasid ticks are known as soft ticks, feed secretively for brief periods and are rarely seen (Fig. 1.3). Soft ticks are known to progress through several (2–7) instars in the nymphal stage prior to molting to the adult reproductive phase; the number of nymphal stages is dependent on species, host availability, and climatic and/or environmental factors. There is little difference in appearance between male and female soft ticks. Tick hosts include mammals, birds, amphibians, and reptiles (Keirans and Durden 2005). Ticks are usually active during summer months and the geographic range and distribution of ticks has increased due to climate change (Ogden et al. 2005).

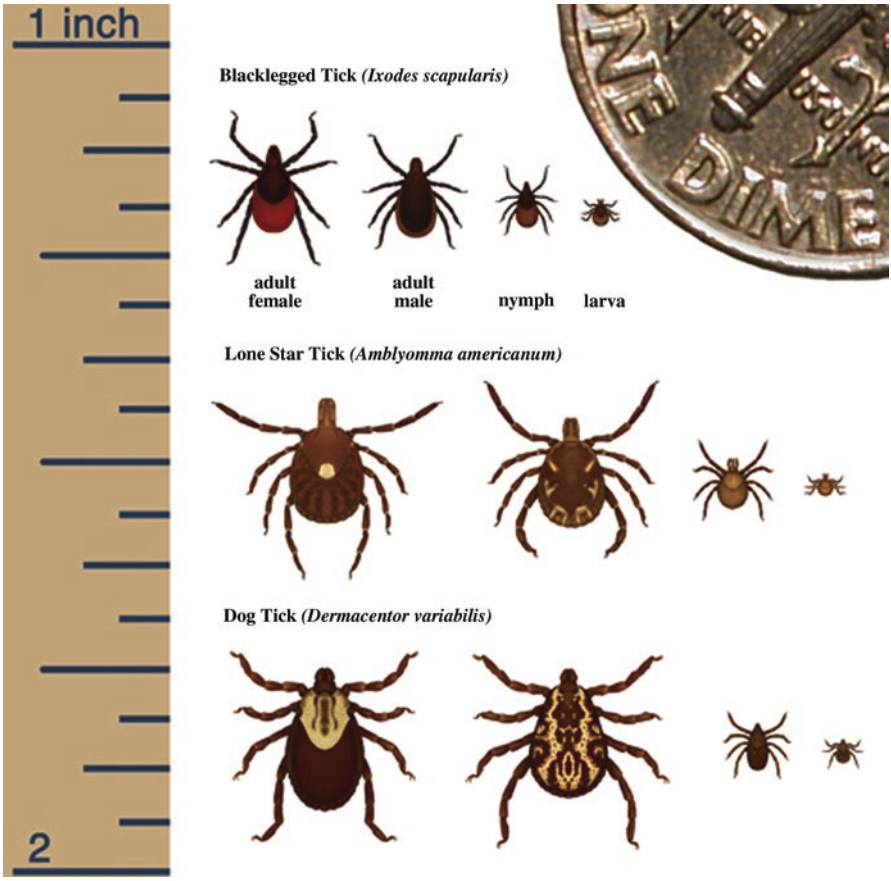

Fig. 1.1 Relative sizes of several ticks at different life stages. Credit: Centers for Disease Control and Prevention

Fig. 1.2 Female blacklegged (deer tick), *Ixodes scapularis* with its abdomen engorged with a host blood meal. Credit: CDC/Dr. Gary Alpert—Urban Pests—Integrated Pest Management (IPM)

Fig. 1.3 Dorsal view of the "soft tick" *Carios kelleyi* (Bat Tick). Credit: CDC/Jim Gathany/William L. Nicholson

12.2 Tick Feeding

Hard ticks parasitize vertebrate hosts for several days or more, and they must attach firmly enough to their host's skin to remain in place in spite of mechanical perturbations associated with various activities, including grooming of the host. The three main structures of ticks involved in feeding and providing a transmission pathway for microbes include the salivary glands, ventral hypostome, and the chelicerae. One of the important functions of the salivary glands relates to attachment. They excrete a proteinaceous cement that helps secure the hypostome of the tick so it is more secure in the body of its host (Anderson and Magnarelli 2008). It also excretes compounds that prevent clotting by slowing platelet aggregation and helps the tick evade the immune system by preventing neutrophil aggregation and T-cell response (Ribeiro 1989). These glands also help regulate water balance in ticks (Bowman and Nuttall 2008). The paired salivary glands are important not only to the function of feeding, but they are also involved in the transfer of microbial pathogens into the host.

The ventral hypostome is a plate armed ventrally with rows of spine-like denticles. At the center of the fixed ventral hypostome is a deep groove that functions to channel blood flow from the host to the tick's mouth and also to channel saliva from the tick's mouth to the host. They function to set the organism firmly in the skin for those ticks that do not secrete cement from their salivary glands (Richter et al. 2013). The chelicerae are paired shafts that have bundles of hook-like structures on their ends. They function to dig into the skin of the host and then pry the skin open so that the hypostome can enter and the feeding process can begin (Richter et al. 2013). They are the mobile component of the feeding apparatus. An overview of tick feeding and pathogen transmission mechanism is shown in Fig. 1.4.

12.3 Tick Life Cycle and Behavior

Ticks are present all over the world (Keirans and Durden 2005). However, they are most diverse in tropical and sub-tropical climates (Anderson and Magnarelli 2008). There is clear distinction in the ecology of the two major tick groups. The hard ticks

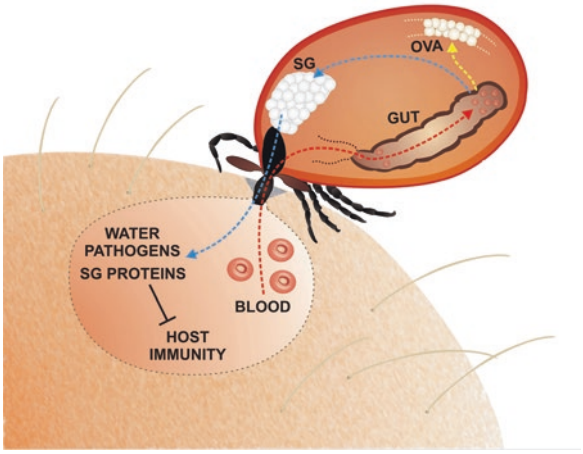

Fig. 1.4 An overview of tick feeding and pathogen transmission mechanism. The pathogen transmission is tightly linked with physiology of blood feeding and tick innate immunity. Ingested blood meal is accumulated in the midgut content. Hemoglobin and other proteins are taken up by the tick midgut cells and digested intracellularly in the digestive vesicles. Liberated amino acids and other compounds are transported to the peripheral tissues and ovaries, supplying mainly egg development. The blood meal is concentrated by reabsorption of excessive water, which is spitted back into the wound by the action of salivary glands. Tick saliva contains a great variety of anticoagulant, immunomodulatory, and anti-inflammatory molecules that facilitate pathogen acquisition and transmission (Credit: Hajdušek et al. 2013)

(*Ixodidae*) spend most of their life on host organisms feeding on blood; whereas, the soft ticks (*Argasidae*) only feed on host blood at night, and spend most of their day hiding in stable microclimate nesting sites (Bogitsh et al. 2005). The important characteristics of these nesting sites include low light and stable microclimates (wind, temperature, humidity). They also must have a high probability of host contact (Anderson and Magnarelli 2008).

During development, the tick goes through four life stages: egg, six-legged larva, eight-legged nymph, and adult. After hatching from the eggs, ticks must eat blood at every stage to survive. Ticks can take up to 3 years to complete their full life cycle, and most will die because they do not find a host for their next feeding. In ticks from *Ixodidae*, this is usually a three host process where they feed and then molt to move onto the next stage (Anderson and Magnarelli 2008). Some species are two-host or even one-host ticks where they feed and molt on the same host. This is generally because the tick lives in an area where host organisms are sparsely distributed (Anderson and Magnarelli 2008; CDC [http://www.cdc.gov/ticks/life_cycle_and_hosts.html]).

Ticks find their hosts by detecting animals' breath and body odors, or by sensing body heat, moisture, and vibrations. Some species can even recognize a shadow. In addition, ticks pick a place to wait by identifying well-used paths. Then they wait for a host, resting on the tips of grasses and shrubs. Ticks cannot fly or jump, but many tick species wait in a position known as "questing" (Fig. 1.5). While questing,

Fig. 1.5 A deer tick (blacklegged tick), *Ixodes scapularis* questing on a blade of grass. Image credit: CDC

ticks hold onto leaves and grass by their third and fourth pair of legs. They hold the first pair of legs outstretched, waiting to climb onto the host. When a host brushes the spot where a tick is waiting, it quickly climbs aboard. Some ticks will attach quickly and others will wander, looking for places like the ear, or other areas where the skin is thinner (Source: CDC).

The vast majority of ticks do not commonly use humans as their host organism. Only 33 of the 878 known species of ticks are known to commonly feed on people (Anderson and Magnarelli 2008), and only 222 of the 878 known species have been documented to feed on humans at all (Anderson and Magnarelli 2008). The difference between these two numbers is somewhat attributed to people coming into close proximity with the main host of that species of tick. The tick *Argas monolakensis* has been found to readily feed on humans despite the main host being *Larus californicus Lawrence*, the California gull (Schwan et al. 1992). Thus, a Tick may feed on humans even though that is not its main host. This is also observed in many other species such as *Rhinicephalus sanguineus* (brown dog Tick) and *Ixodes scapularis* (deer tick) (Dantas-Torres et al. 2006; Kilpatrick et al. 2014).

To remove a tick

1. Use fine-tipped tweezers to grasp the tick as close to the skin's surface as possible.
2. Pull upward with steady, even pressure. Do not twist or jerk the tick; this can cause the mouth-parts to break off and remain in the skin. If this happens, remove the mouth-parts with tweezers. If you are unable to remove the mouth easily with clean tweezers, leave it alone and let the skin heal.
3. After removing the tick, thoroughly clean the bite area and your hands with rubbing alcohol, an iodine scrub, or soap and water.
4. Dispose of a live tick by submersing it in alcohol, placing it in a sealed bag/container, wrapping it tightly in tape, or flushing it down the toilet. Never crush a tick with your fingers (Source: CDC).

12.4 *Other* Rickettsiales *Vectors*

Ticks are not the only vectors for transmission of bacteria of the order *Rickettsiales*. Other closely related ectoparasites such as lice and mites have been known to transmit rickettsial diseases, but ticks have a greater contribution to human infection than either of these.

Mites (Fig. 1.6) are closely related to ticks. The Trombiculid mite (genus *Leptotrombidium)*, especially the larval stages (commonly called chigger) is the major vector involved in transmission of several rickettsial diseases. They are involved in infecting humans with pathogenic O*rientia tsutsugamushi* that causes Scrub Typhus (Yamashita et al. 1994). Transmission of *Orientia* to the rodent host or the human incidental host occurs during feeding chigger stage of mites. Vertical or transovarial transmission appears to be essential to the maintenance of the infection in nature; thus, the mite serves as both the vector and the reservoir. As the larval stage is the only parasitic stage of *O. tsutsugamushi*, to maintain disease transmission, it is necessary for *O. tsutsugamushi* to be transmitted transstadially through the nymph and adult stages and transovarially transmitted through the eggs to the progenies. The efficiency of transmission of *Orientia* by infected chiggers is important in determining how the disease is maintained in nature (Shin et al. 2014).

Louse (Fig. 1.7) infestation, called pediculosis, is very contagious and is easily transmitted by close body-to-body contact or contact with infested linen, brushes, or clothes, according to the species of louse. Pediculosis corporis, caused by body lice (*Pediculus humanus*), is a major public health concern. It is strongly associated with close body-to-body contact, and occurs only when clothes are not changed or washed regularly. These conditions are more prevalent in individuals living in crowded and unhygienic environments, such as refugee camps or shelters for the homeless. Body lice are known to transmit epidemic typhus, caused by *R. prowazekii*. The mortality rate of epidemic typhus varies from 0.7 to 60 % for untreated cases. Lice become infected with *R. prowazekii* when they feed on bacteremic individuals; however, lice die within 1 week after becoming infected. Humans with

Fig. 1.6 Photograph of a mite, a member of the Class *Arachnida*, Order *Acari*. Image credit: CDC

Fig. 1.7 Body lice live and lay eggs on clothing and only move to the skin to feed. Image credit: CDC

Fig. 1.8 The Oriental rat flea, *Xenopsylla cheopis.* Image credit: CDC

self-limiting infections that fail to clear the bacteria and exhibit bacterial persistence in adipose tissue endothelial cells constitute the main reservoir of *R. prowazekii*. Under stress, infection recrudescence can occur years after the primary infection, resulting in a relatively mild bacteremic illness called Brill–Zinsser disease (Badiaga and Brouqui 2012).

Flea-borne infections are emerging or re-emerging throughout the world, and their incidence is on the rise (Bitam et al. 2010). Fleas (Fig. 1.8) (class: Insecta, order: Siphonaptera) are small, laterally flattened, wingless, and highly specialized insects about 2–10 mm in length. They have thin, flattened bodies and backward-directed spines on their legs and bodies that facilitate forward movement through fur, hair, or feathers and prevent them from being easily dislodged. They have strongly developed hind legs that permit them to jump up to 150 times their own body length. There is no flea specific to humans, and only a fraction of all fleas come into contact with humans on a regular basis. Many fleas, however, associate

with domesticated animals, and may thus have an economic, rather than direct effect on humans and their health. Fleas are involved in the transmission of *Rickettsia typhi* (causes murine typhus) and *Rickettsia felis* (causes cat flea typhus).

R. typhi is a member of the typhus group rickettsiae. The disease is characterized by headache, rash, and fever and occurs worldwide in a variety of environments. The oriental rat flea *Xenopsylla cheopis* is the main vector of *R. typhi.* The classic cycle of murine typhus involves rats (*Rattus norvegicus* and *R. rattus*) as reservoirs, and their fleas. However, other vertebrate hosts, such as house mice, shrews, opossums, skunks, and cats, which live in or enter rat-infested buildings and human habitations, may be involved in the epidemiology of murine typhus. Although *X. cheopis* is considered the major vector of murine typhus, natural infection with *R. typhi* has been reported in other flea species (Christou et al. 2010). *R. typhi* infects endothelial cells in mammalian hosts and mid-gut epithelial cells in the flea host. It is passed in the flea's feces, and transmission to humans is by fecal contamination (Bitam et al. 2010).

The cat flea (*Ctenocephalides felis*) serves as the primary vector and reservoir of *R. felis. R. felis* transmission is primarily vertical (transovarial and transstadial) within a flea population, rather than horizontal between fleas through a bloodmeal. Infection by *R. felis* has been attributed to flea saliva rather than feces (Giudice et al. 2014).

The distribution of vectors and associated pathogen transmission rates can be affected by changes in the ambient temperature and climate. Such climate changes will cause local vector populations to migrate to more favorable climates alongside vertebrate hosts and also alter the life cycle duration of vectors. Unchecked expansion of vectors would potentially adversely affect human health. There is a need to be vigilant in identifying both current and emerging vector-borne diseases in the environment. Influence of climate change on temperature levels may be far-reaching. Not only could it affect arthropod life cycles but also human activities as well. Use of the land in affected areas will be influenced and long-term activities such as farming and tourism will indirectly affect transmission of arthropod-borne diseases. Better understanding of endemic diseases will equip doctors, veterinarians, and public health officials with the information needed to prevent outbreaks and provide proper treatment (Abdad et al. 2011).

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Chapter 2
Molecular Epidemiology of Rickettsial Diseases

Marina E. Eremeeva

1 Introduction

A contemporary definition of molecular epidemiology emphasizes the integration of the principles and practices of traditional epidemiology with the powerful tools of molecular biology to enhance the number of topics amenable to epidemiological analysis. For example, this new field includes studying molecular effects of environmental variables, and host and microbial genetic risk factors on the etiology, transmission and distribution of diseases, and improved disease surveillance. These new types of studies for any group of diseases or agents including the rickettsioses are very dependent on advances in and novel applications of molecular methods which are frequently used for molecular taxonomy and phylogeny; however, these studies are quite different from molecular epidemiology even though they also depend on molecular advances. Taxonomy is the science of classification of organisms into naturally related groups based on a consistent collection of common but not identical features, and those can be polygenic or epigenetic rather than exclusively based on gene sequences. In contrast, in phylogenetic studies the lines of descent or evolutionary relationships of a group of organisms are inferred primarily by the pattern of gain and loss or mutation of genetic properties. Therefore, any application of particular molecular tools to type or classify pathogenic organisms and their near relatives does not by itself constitute molecular epidemiology. On the other hand, the use of these tools to obtain molecular data which may be used to infer trends in incidence, unexpected origins, spread or transmission of a disease in an outbreak and the true prevalence of particular diseases at the population level are forms of molecular epidemiology.

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Fig. 2.1 Portrait of Dr. Nataliya Balayeva (1926–1995)

The concept of molecular epidemiology in the context of rickettsial diseases was first formally used by Dr. Nataliya Balayeva of the Gamaleya Institute of Microbiology and Epidemiology in Moscow (Fig. 2.1). She proposed that the application of contemporary genetic methods to distinguish circulating isolates would aid in understanding observed changes in the incidence and temporal and spatial distribution of rickettsioses (Balayeva 1989). At the time this approach was very innovative since traditional surveillance of rickettsial diseases relied primarily on records of clinical data and patient exposure history with or without discriminatory serological data (Zdrodovskii and Golinevitch 1960). While those tests could distinguish different groups of distantly related agents, their further immunological and biological differentiation was heavily dependent on the rare cases of isolation of rickettsiae from clinical specimens or case-associated environmental samples or pathological findings highly typical for those diseases (Walker and Peacock 1988). Thus disparate diseases could be lumped together unwittingly and lead to false epidemiological conclusions.

Several detailed reviews have summarized the most recent information regarding the diversity of currently known rickettsial pathogens, their spatial distribution, their defined or suggested associations with known and emerging rickettsioses, and their phylogeography (Parola et al. 2005, 2009, 2013; Labruna 2009). This chapter will describe the different tools used in both the classical and molecular epidemiology of rickettsioses. Primary emphasis will be placed on selected examples of specific applications of those molecular tools in various recent epidemiological investigations from this large and expanding field of rickettsiology. Finally, prospects for future improvements in the molecular epidemiology of the rickettsioses are considered.

2 Epidemiology of the Classic Rickettsioses Found in the USA in the Pre-molecular Era

Historically, identification and diagnosis of rickettsial diseases relied upon clinical recognition of diseases confirmed by serological testing, and rarely, when possible, isolation of rickettsiae from patient blood or associated environmental samples and

pathological examination of fatal cases (Zdrodovskii and Golinevitch 1960; Walker and Peacock 1988). In the USA, the original national case definition used for surveillance of Rocky Mountain spotted fever (RMSF) was developed in 1990 (Case definition 1990). It underwent several refinements and modifications, leading to a version which actually recognizes diverse spotted fever rickettsioses including RMSF (Fig. 2.2) (CSTE 2010). The latest case definition was developed to acknowledge the existence a plethora of nosological entities presenting with similar clinical manifestations but caused by distinct etiological agents across the USA (Table 2.1). Below we describe four classic rickettsioses known in the USA in the pre-molecular

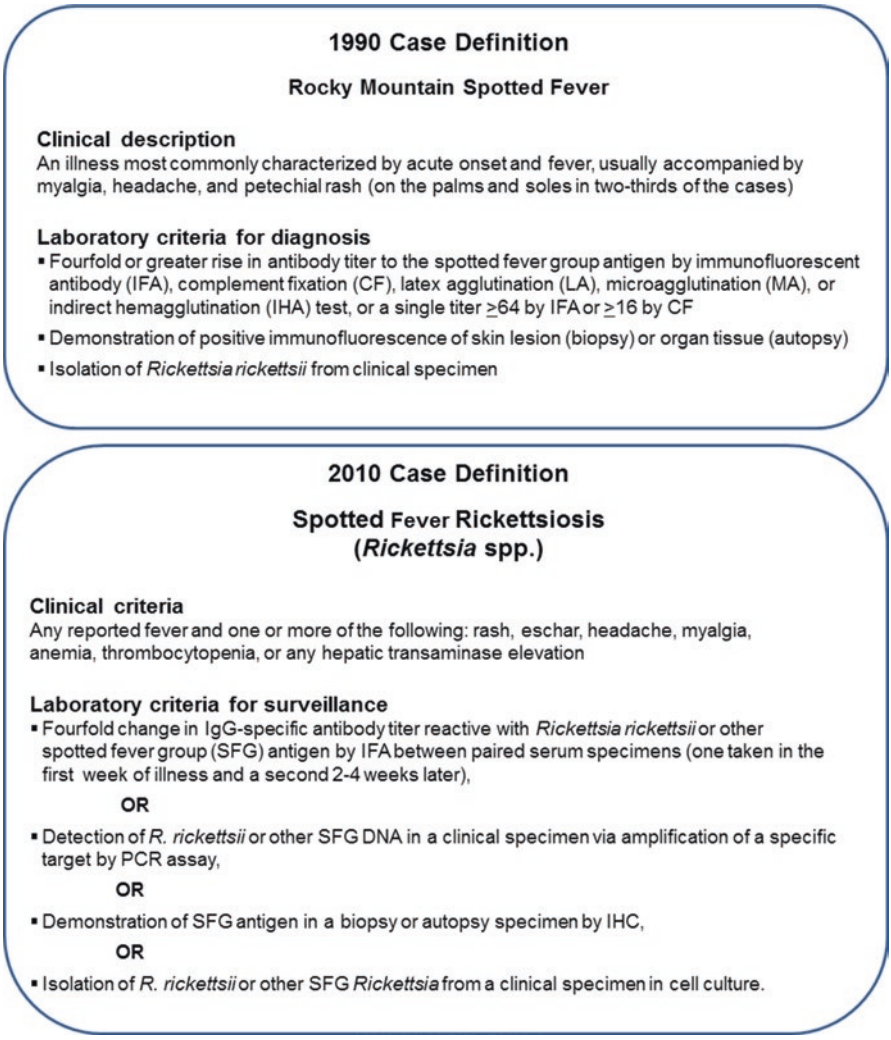


Fig. 2.2 1990 and 2010 Case definitions for reporting Rocky Mountain spotted fever and spotted fever group rickettsioses in the USA

Table 2.1 Rickettsioses, their etiological agents and primary vectors in the USA

Disease	Agent	Vector
Rocky Mountain spotted fever	*Rickettsia rickettsii*	*Dermacentor andersonii, D. variabilis, Rhipicephalus sanguineus, Amblyomma canjennense*
R. parkeri rickettsiosis	*Rickettsia parkeri*	*Amblyomma maculatum*
R. philipii rickettsiosis, (Pacific Coast Tick Fever)	*Rickettsia philipii*	*Dermacentor occidentalis*
R. felis infection	*R. felis*	*Ctenocephalides felis*
R. massiliae rickettsiosis[a]	*Rickettsia massiliae*	*Rhipicephalus sanguineus*
Murine typhus	*Rickettsia typhi*	*Xenopsylla cheopis*
Sylvatic typhus	*Rickettsia prowazekii*	Ectoparasites of flying squirrels
Rickettsialpox	*Rickettsia akari*	*Liponyssoides sanguineus*
African tick-fever[b]	*Rickettsia africae*	*Amblyomma variegatum*

[a]*R. massiliae* was detected in the US brown dog ticks from several locations (Eremeeva et al. 2006a, b; Beeler et al. 2011; Fornadel et al. 2013); however, human cases were not identified.
[b]African tick-bite fever is not endemic to the USA; however, it may occur in US residents who have travelled to endemic regions in Africa and the Caribbean.

era followed by a discussion of the application and impact(s) of the various serological methods used in classic approaches to rickettsial epidemiology. We also note how improvements in some of those methods can contribute to a contemporary role for them in molecular epidemiology.

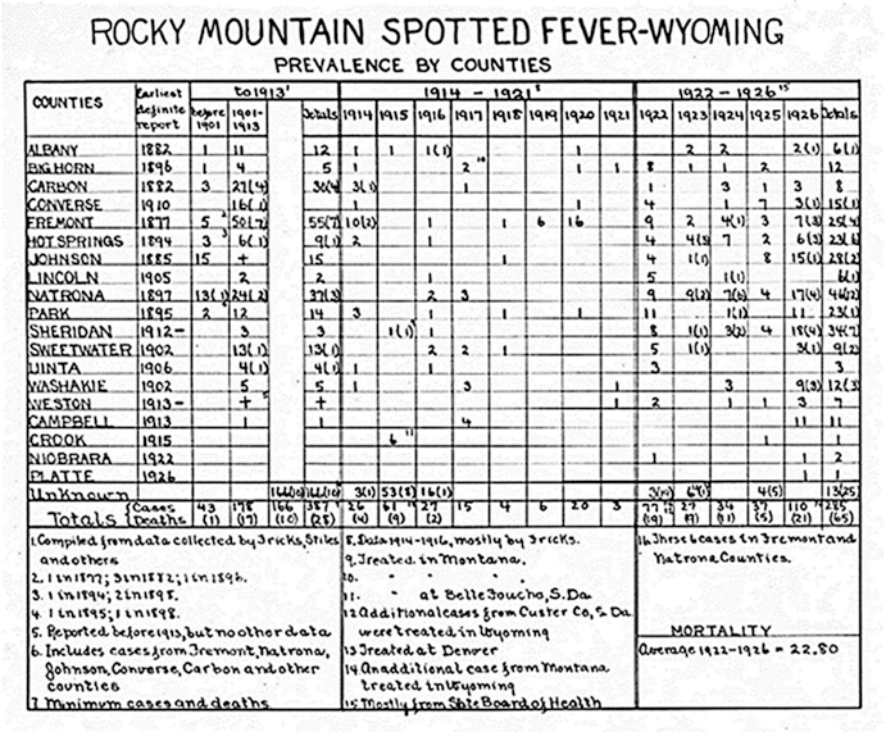
2.1 Classic Endemic Diseases in the United States and Epidemiological Concepts

2.1.1 Rocky Mountain Spotted Fever

Rocky Mountain spotted fever was the earliest recognized endemic rickettsiosis in the USA. It was described as a clinical entity in 1899, and was first recognized in the north-western Rocky Mountains and especially known in the Bitterroot Valley of Montana. The hallmark symptoms of RMSF — the blue-black rash, high fever and delirium, — and a high mortality rate, especially among healthy young adults produced anxiety in prospective residents about significant risks for an early and horrible death from this disease. Practical measures to address the understanding, prevention, and control of RMSF were initiated by the Montana State Board of Health in 1902 (Harden 1990). Originally it was thought that the disease was limited to the Rocky Mountain region; however, subsequently cases were also recognized in eastern and southeastern parts of the country as well as other parts of the West. Since 1971, more than 97 % of all cases have occurred in the mid Central parts of the US, from Oklahoma to North Carolina and adjacent states (Burgdorfer 1988); this trend remains unchanged to the present (Adjemian et al. 2009). The disease

became nationally notifiable in the USA due to its morbidity and mortality. Presently, it is a reportable disease in Canada, throughout Mexico and Central America to Brazil and Argentina. During the early days of surveillance of RMSF, particularly, in the pre-antibiotic era, a much greater case-fatality rate was reported relative to disease incidence compared to the present era (Fig. 2.3).

The introduction of effective antibiotics for treatment of RMSF resulted in a significant reduction of mortality (Fig. 2.4). However, the late 70s were characterized by an increase in the prevalence of rickettsial diseases across the globe: this was also observed for RMSF. After a slight decline in 1990, there has been a continuous increase through the present. The current upward trend in morbidity of rickettsial infections has been characterized by a steady decline in the case-fatality rate. Some of this decline may be due confusion of RMSF cases with other SFG rickettioses when serology was the sole basis for diagnosis of the infection. From the public heath prospective, reduced mortality due to RMSF is the ultimate goal; however, understanding and managing current trends in disease morbidity remains a serious task to enable control and prevention of extended hospital stays. This situation has become a source of extended discussion in the literature (Raoult and



ROCKY MOUNTAIN SPOTTED FEVER-WYOMING

PREVALENCE BY COUNTIES

COUNTIES	Earliest definite report	to 1913[1]			1914 – 1921[2]								1922 – 1926[15]					
		before 1901	1901-1913	Totals	1914	1915	1916	1917	1918	1919	1920	1921	1922	1923	1924	1925	1926	Totals
ALBANY	1882	1	11	12	1	1	1(1)				1			2	2		2(1)	6(1)
BIG HORN	1896	1	4	5	1			2[14]			1	1	8	1	1	2		12
CARBON	1882	3	21(4)	34(4)	3(1)			1					1		3	1	3	8
CONVERSE	1910		16(1)		1						1		4		1	7	3(1)	15(1)
FREMONT	1877	5[3]	50(7)	55(7)	10(2)		1		1	6	16		9	2	4(1)	3	7(3)	25(4)
HOT SPRINGS	1894	3[3]	6(1)	9(1)	2		1						4	4(3)	7	2	6(3)	23(6)
JOHNSON	1885	15	+	15					1				4	1(1)		8	15(1)	28(2)
LINCOLN	1905		2	2			1						5		1(1)			6(1)
NATRONA	1897	13(1)[4]	24(2)	37(3)			2	3					9	9(2)	7(3)	4	17(4)	46(12)
PARK	1895	2	12	14	3		1		1		1		11		1(1)		11	23(1)
SHERIDAN	1912–		3	3		1(1)[5]	1						8	1(1)	3(2)	4	18(4)	34(7)
SWEETWATER	1902		13(1)	13(1)			2	2	1				5	1(1)			3(1)	9(2)
UINTA	1906		4(1)	4(1)	1		1						3					3
WASHAKIE	1902		5	5	1			3				1			3		9(3)	12(3)
WESTON	1913–		+[5]	+								1	2		1	1	3	7
CAMPBELL	1913		1	1				4									11	11
CROOK	1915					6[11]										1		1
NIOBRARA	1922												1				1	2
PLATTE	1926																1	1
Unknown				144(9)[7] 44(10)[7]	3(1)	53(8)	16(1)						3(1)	6(1)		4(5)		13(25)
Totals {Cases / Deaths}		43 (1)	198 (17)	166 (10) 387 (28)	26 (4)	61[9] (9)	27 (2)	15	4	6	20	3	77[13] (19)	29 (8)	34 (9)	37 (5)	110[16] (21)	285 (65)

1. Compiled from data collected by Dricks, Stiles and others
2. 1 in 1917; 3 in 1872; 1 in 1894.
3. 1 in 1894; 2 in 1895.
4. 1 in 1895; 1 in 1898.
5. Reported before 1913, but no other data
6. Includes cases from Fremont, Natrona, Johnson, Converse, Carbon and other counties
7. Minimum cases and deaths
8. Data 1914-1916, mostly by Dricks.
9. Treated in Montana.
10. " " " "
11. " at Belle Fouche, S. Da.
12. Additional cases from Custer Co, S. Da. were treated in Wyoming
13. Treated at Denver
14. An additional case from Montana treated in Wyoming
15. Mostly from State Board of Health
16. Three cases in Fremont and Natrona Counties.

MORTALITY

Average 1922-1926 = 22.80

Fig. 2.3 Prevalence of Rocky Mountain spotted fever in Wyoming, 1913–1926 (by county). Source: the Rocky Mountain Laboratories, National Institutes of Health; http://mtmemory.org/cdm/singleitem/collection/p16013coll2/id/169/rec/188

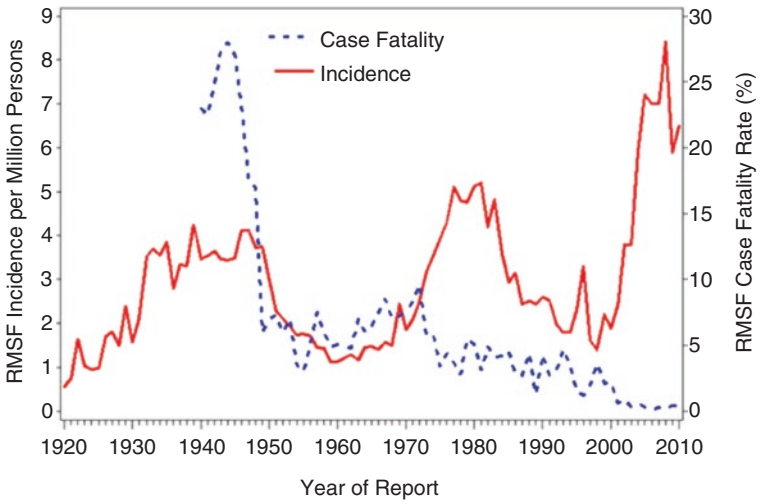


Fig. 2.4 National incidence and case-fatality rate of Rocky Mountain spotted fever in the USA. Source www.cdc.gov

Parola 2008; Openshaw et al. 2010), resulting in identification of several objective and subjective causes leading to these observations. Beside the passive system of disease surveillance in the USA, a lack of agreed standards for interpretation of the test results (the majority of which are subjective serological methods based on detection of cross-reacting antigens by serum antibodies) and changes in the availability and technological properties of laboratory detection and diagnostic tools are confounding factors (Openshaw et al. 2010). True or perceived changes in the geographic distribution of traditionally diagnosed rickettsioses and description of new etiological agents co-circulating in these areas may be important factors contributing to the current uncertain epidemiological situation both in the US and worldwide (Parola et al. 2009, 2013). This is particularly illustrated by the recent finding that the highly prevalent carriage of *Rickettsia amblyommii* by the lone star tick, *Amblyomma americanum*, may actually contribute to the total number of cases previously counted as RMSF in Tennessee and elsewhere (Moncayo et al. 2010). Because the agent may cause mild illness, it could contribute to the apparent decline of fatal cases of RMSF in the USA (Dasch et al. 2001, 2006; Billeter et al. 2007; Apperson et al. 2008; Nicholson et al. 2009).

2.1.2 Rickettsialpox

In contrast to RMSF, rickettsialpox has the briefest history between its initial recognition, characterization, and effective control (Paddock and Eremeeva 2007). Originally described in an urban setting in New York City in 1946 (Shankman 1946), the disease received considerable attention during the late 1940s and early

1950s when over 500 clinical cases were diagnosed in the New York City. The disease remains reportable in the city of New York (Reporting Diseases & Conditions). A second large and nearly contemporaneous outbreak of rickettsialpox occurred in 1949 in Ukraine where the disease was extensively studied throughout the 1950s (Kulagin 1952); however, interest in this relatively benign rickettsioses slowly waned during subsequent decades despite increasing records of its global distribution (Choi et al. 2007; Zavala-Castro et al. 2009; Renvoise et al. 2012; Denison et al. 2014). *Rickettsia akari*, the etiological agent of rickettsialpox, is transmitted by mites infesting peridomestic mice and rats. Rodent eradication programs without simultaneous vector control or diseases in rodents leading to die-offs may lead to inner city outbreaks of rickettsialpox because the mites are known to seek a new host and will attack people more frequently in the absence of significant rodent populations (Paddock and Eremeeva 2007).

2.1.3 Murine Typhus

Endemic or flea-borne murine typhus is caused by *Rickettsia typhi*. It was originally regarded clinically as a modified form of classic louse-borne typhus so it was often not recognized as a separate disease until definitive eco-epidemiological and animal studies were performed by the 1930s (Traub et al. 1978; Woodward 1982). The disease was endemic across most of the US, especially along the Atlantic and Gulf Coasts and south eastern United States. It was a nationally notifiable disease and morbidity reached its maximum rate of 5,500 annual cases in 1943–1944. Following broad application of DDT and rodenticides during rodent control campaigns, the disease was nearly eradicated (Love and Smith 1960). At present, murine typhus is only reported in Texas, California, and Hawaii (Civen and Ngo 2008); however, in each of these states and elsewhere in the USA, the true prevalence is not known because it is not nationally reportable and cases are rarely investigated epidemiologically and ecologically outside of these three states. This deficit of attention is of particular concern due to the emerging status of this illness on a global scale (Walter et al. 2012). The occurrence of other diverse flea-borne rickettsiae that have now been recognized by molecular tools (see below in this chapter) is a confounding factor as much as for SFG rickettioses in the USA with RMSF (Jiang et al. 2013; Mediannikov et al. 2015).

2.1.4 Sylvatic Typhus

As in the rest of the world, the USA was affected by outbreaks of classic louse-borne typhus prior to elimination of body lice. The actual morbidity of this disease in the USA historically is very hard to estimate due to clinical misidentification with typhoid fever (Agnew 1890); however, some of the largest and most recent known outbreaks occurred in Philadelphia (Agnew 1890; Curtin 1890), New York (Rosen 1972), and Baltimore (Cordell 1903). Description of the sporadic outbreaks arising

from recrudescent Brill-Zinsser disease was another epidemiological milestone for better understanding the sources of epidemic typhus affecting populations in the USA (Zinsser 1934); however, these were mostly low impact events because of the very low rates of body lice in modern US populations. In contrast, discovery of sylvatic typhus as an extra-human reservoir of *Rickettsia prowazekii* restored public health interest in this disease (Bozeman et al. 1975). This discovery is a perfect example illustrating both the utility and limitations of classic laboratory methods used in rickettsiology before molecular era tools became available. Serological evidence for enzootic typhus group infection of flying squirrels was first found near Montpelier, Virginia in 1963 during studies of the ecology of RMSF in small mammals (Bozeman et al. 1975). Complement fixation tests, using species-specific corpuscular antigens prepared from yolk sacs infected with epidemic and murine typhus rickettsiae and *R. canadensis,* were carried out on specimens collected in 1968. The results showed that either *R. prowazekii* or *R. canadensis* or another organism antigenically related to both was present in the squirrels and their flea and louse ectoparasites. Complement fixation tests with sera from 30 squirrels collected near Ashland, Virginia in the spring of 1972 showed that 42% of the animals were exposed to the agent. Subsequent testing using indirect immunofluorescence assay (IFA) confirmed these preliminary findings (Bozeman et al. 1975). The rickettsial agents were then isolated from flying squirrels and their ectoparasites, and were found to be identical to *R. prowazekii* based on the classic toxin neutralization assay (Bozeman et al. 1975), and their biology, protein, and iso-enzyme composition profiles (Woodman et al. 1977; Dasch et al. 1978). Genetic identification of these isolates as *R. prowazekii* was first done in 2000, subsequently confirmed in 2007 using multi-locus sequence typing and corroborated by whole genome sequences analysis (Moron et al. 2000; Ge et al. 2007; Bishop-Lilly et al. 2013). More than 40 human cases of sylvatic typhus have been confirmed using classical serological methods to distinguish antibodies to *R. typhi* and *R. prowazekii* and environmental investigations for the presence of infected squirrels near the cases. Availability of this genetic information combined with molecular methods of investigation enabled direct identification of an enzootic cycle of *R. prowazekii* in Pennsylvania Boys Camp in 2004–2006, and determined the sylvatic typhus etiology of this outbreak, originally misidentified as flu and mononucleosis, and confirmed that close contact with nesting squirrels and ectoparasites is required for disease transmission (Chapman et al. 2009).

2.2 *Improved Serological Tools and Their Important Role in Investigating the Epidemiology of Rickettsial Diseases*

Early diagnosis of rickettsial diseases was primarily based on recognition of clinical symptoms and associated eco-epidemiological observations. Later a battery of serological methods using whole cell antigens to detect agglutinating antibodies or antibodies bound to erythrocytes became available. This arsenal included the Weil-Felix reaction permitting detection of group-cross-reactive IgM type antibodies, microagglutination tests, latex-agglutination tests, complement fixation

Table 2.2 Molecular methods used in epidemiological studies and their applicability (usefulness) for the field of rickettsial diseases

Methods	Techniques	Applications
Detection of antibodies	1. Microimmunofluorescence assay	1. Confirming exposure to rickettsial pathogens
	2. Enzyme-linked immunosorbent assay	2. Serotyping
	3. Immuno-peroxidase assay	3. Differential identification of exposure
	4. Western blotting	
Detection of antigens	1. Culture isolation	1. Pathogen isolation
	2. Immunohistochemistry	2. Pathogen detection
	3. Immuno-peroxidase assay	
	4. Mass-spectroscopy	
Detection of nucleic acid	1. PCR-based detection assays	1. Detection of the pathogen-specific target
		2. Multi-locus gene typing
		3. Restriction fragment gene analysis
		4. Multi-locus intergenic region typing
		5. Plasmid profile
		6. Typing based on variable tandem repeat region typing
Detection of gene expression	1. Reverse-transcription PCR	1. Confirmation of the agent viability and accumulation of pathological changes
	2. Cytokine assays	2. Identification of host responses
	3. Microarray techniques	

tests, and indirect hemagglutination tests for different groups or species of *Rickettsia*. While very useful at the time and still currently available in some countries around the world, all of these methods suffer from various drawbacks ranging from low sensitivity to a requirement for large amounts of relatively purified rickettsial whole cell antigen, and inconsistent results when applied to different rickettsial infections (Elisberg and Bozeman 1979). The most frequently used rickettsial diagnostic methods are listed in Table 2.2; their descriptions are provided in the following sections.

2.2.1 Indirect Immunofluorescence Assay

Indirect immunofluorescence assay (IFA) is the current gold standard for serodiagnosis of rickettsial diseases (Philip et al. 1976; Elisberg and Bozeman 1979). The details of this method are described in other sections of this book. However, it is important to emphasize that it permits detection of both IgM and IgG type antibodies. The IFA can allow simultaneous detection and differentiation of antibodies against several

species of rickettsiae, especially when it is coupled with cross-adsorption (depletion) procedures targeted to remove species-specific or cross-reacting antibodies. Cross-reactive IFA titers obtained with gamma-specific IgG conjugates are used for primary diagnostic confirmation of acute rickettsial illness. IFA sensitivity is 94–100 % by 14 days of illnesses such as RMSF; however, mild rickettsioses such as *Rickettsia parkeri* rickettsiosis may not exhibit significant titers until after 25–28 days following onset of acute illness (Biggs et al. 2016). IFA differential diagnosis is based on serum recognition of specific epitopes on the lipopolysaccharide (LPS — typhus vs. spotted fever groups) and major protein antigens (namely OmpA and OmpB) of rickettsiae. These antibodies clearly differ in their affinity and specificity during the course of infection and vary between the individuals and animal species. A modification of this method using hyperimmune polyclonal mouse sera to the whole cell rickettsial antigens was developed and widely used for serotype-based identification of rickettsial isolates (Philip et al. 1978). This approach is based on the fact that mice react by preferentially synthesizing antibody to the immunodominant species-specific epitopes of surface antigens (OmpA and OmpB) possessed by rickettsiae; quantitative parameters based on the relative reactivity of homologous and heterologous sera to different *Rickettsia* species antigens are used. Accordingly, it was proposed that a fourfold difference in titers determined for reactivity of mouse immune serum with homologous and heterologous antigens is the minimum difference required to distinguish two different serotypes or species of rickettsiae (Philip et al. 1978). Similar quantitative differences are sometimes assumed to always exist when testing sera of human and other animals (Pinter et al. 2008; Tamekuni et al. 2010; Lado et al. 2015); however, systematic experiments confirming applicability of this approach outside of murine system have not been conducted, but human and many animal sera may exhibit much less species-specific reactivity than mouse sera and recognize cross-reactive epitopes on LPS and the outer membrane proteins. This is particularly significant, because human sera can even exhibit significant cross-reactivity between typhus and spotted fever groups of rickettsiae, and even the IgM with other bacteria including *Legionella* and *Proteus* species (Raoult and Dasch 1995); hence, IgG is preferred for measurement of seroconversions.

2.2.2 Western Blotting

Western blotting is a sophisticated and qualitative serological procedure sometimes permitting direct differential identification of rickettsial exposure (and thus the etiology of their infection) and differentiation of false-positive from true positive results. Western blotting is very sensitive and the sole general method besides cross-absorption and blocking assays (coupled with IFA or other serological methods) which can identify the source of infections by detecting the specificity of antibody reactions with the rickettsial OmpA and OmpB proteins. It too is more species-specific following antigen cross-absorption. In the case of some SFG infections, this method appears to be more sensitive compared to IFA due to early detection of antibodies reactive with group-specific LPS but this provides no species-specific

diagnosis (La Scola and Raoult 1999). One significant drawback is that western blotting is time-consuming and dependent on availability of a complete collection of purified rickettsial antigens; nevertheless it is used very successfully by established reference laboratories for confirming the etiology of several rickettsioses as well as for differential diagnosis of known diseases when specimens for molecular identification are not available (La Scola et al. 2000; Raoult and Paddock 2005). These examples include confirmatory diagnosis of clinical cases due to *R. philipii* and a possible etiological role of *R. massiliae* in canine rickettsiosis in California, respectively (Shapiro et al. 2010; Beeler et al. 2011).

2.2.3 Immunohistochemical Methods

Immunohistochemical methods (IHC) are used for direct detection of rickettsial cells in sections prepared from fixed tissues. This procedure was originally developed and is most frequently employed in examination of tissues autopsied from fatal cases but it can be applied to other organ biopsies, including eschar and skin rash biopsies (Procop et al. 1997; Demma et al. 2005; Paddock et al. 2014). Polyclonal antibodies which are group reactive are generally employed for detection but species-specific (e.g., mouse) or cross-absorbed antibodies can be used in parallel if appropriate monoclonal antibodies are not available. Use of this technology coupled with molecular methods has proven to be very informative in the etiological diagnosis of all endemic rickettsioses in the USA manifesting with eschar and/or rash, including infections caused by *R. akari*, *R. parkeri*, *R. philipii*, and *R. rickettsii*, as well as common imported diseases, such as those caused by *R. africae, R. conorii*, and *Orientia tsutsugamushi* (Paddock et al. 2004, 2014; Demma et al. 2005; Paddock and Eremeeva 2007; Shapiro et al. 2010; Edouard et al. 2013; Khrouf et al. 2016). Further improvements in IHC analysis are largely dependent upon development and availability of monospecific or monoclonal antibodies with defined specificity and affinity as well as multicolor assays that may permit simultaneous detection of mixed infections. Unfortunately, this method is relatively infrequently used as it is limited to a few specialty laboratories due to the requirements for professional pathology qualifications, quality instrumentation for sample processing, and specific IHC reagents needed to perform the pathological evaluations.

3 Molecular Genetic Tools in Rickettsiology

The use of molecular genetic tools in rickettsiology and particularly in epidemiology has a relatively short history (Balayeva 1989; Eremeeva 2012), mostly due to the fact that obtaining original genetic information was impeded by the obligate intracellular lifestyle of these microorganisms. Nevertheless, *R. prowazekii* was the seventh bacterium whose full genome sequence was obtained (Andersson et al. 1998) and genomes of many well-studied *Rickettsia* species have been fully

sequenced today. The limitation remaining is that no isolates have been obtained for some diseases and putative new species of *Rickettsia*. Next-generation sequencing approaches can overcome some of these difficulties for uncultivated or uncultivable agents (Gillespie et al. 2012).

3.1 *Analysis of 16S rRNA and 23S rRNA Genes for Identification of* Rickettsia

Although useful for identification of many bacterial species, sequencing of the 16S rRNA gene of *Rickettsia* has provided little ammunition for surveillance and differential diagnosis of most representatives of this genus of microorganisms. In particular, it was determined that genetic diversity among different species of *Rickettsia* ranges from 97.9 to 99.8 % of genetic sequence similarity, and is thus significantly inferior compared to many other microorganisms (Roux and Raoult 1995; Stothard and Fuerst 1995; Fournier et al. 2003). For the most divergent rickettsial agents PCR with conserved eubacterial 16S rRNA gene primers has been important for permitting identification of novel agents which belong to the genus *Rickettsia* (Roux and Raoult 1995) because other genus-conserved primers do not always work. Therefore, detection of the 16S rRNA gene is only infrequently used in diagnostic assays or employed for molecular detection of novel rickettsiae (Lloyd et al. 2011; Giulieri et al. 2012; Hajduskova et al. 2016). It is also a reliable target for identifying *Rickettsia* as a part of microbiome studies in arthropods (Budachetri et al. 2014; Clayton et al. 2015). Other targets have been used in newer quantitative PCR assays based on TaqMan or dye intercalating methodologies (see Sect. 3.3 below).

Unlike many other bacteria genetic typing approaches based on differences in the 16S-23S rRNA gene intergenic region, this is not possible for *Rickettsia* because these genes are separated and located in different parts of the chromosome (Andersson et al. 1995). In contrast, the larger sequence of the rickettsial 23S rRNA gene provides a simple diagnostic target for identification of many *Rickettsia* species; in particular, it has utility for designing species-specific diagnostic assays for so-called outlier species such as *Rickettsia helvetica* (Boretti et al. 2009). PCR amplification and sequencing of the 23S-5S intergenic spacer (IGS) fragment alone or combined with reverse line blot hybridization with species-specific probes may be informative for molecular identification of some species of *Rickettsia* since the 16S-23S rRNA intergenic region approach cannot be used (Lee et al. 2014).

3.2 *PCR Amplification and Gene Targets*

Despite the availability of whole genome sequences for an extended collection of *Rickettsia* species, six conserved genes are those most frequently used targets for detection and identification of these organisms: the 17 kDa protein antigen gene,

citrate synthase gene (*glt*A), and RNA polymerase subunit B (*rpo*B), and three genes encoding for surface exposed protein antigens (*omp*A, *omp*B, and *sca*4). This situation is perpetuated by the current proposed criteria for definition of *Rickettsia* species (Fournier et al. 2003). In particular, use of whole gene or specific fragments of the genus-common *glt*A and *omp*B genes allow reliable discrimination of species of *Rickettsia* in both the typhus and spotted fever groups while *omp*A affords greater discrimination among SFG rickettsiae where it is present (Roux and Raoult 1995; Roux et al. 1996, 1997; Fournier et al. 1998). Accordingly, the range of the sequence variations is 93.3–99.9 % for *glt*A for both typhus and SFG rickettsiae and 55.4–98.8 % for SFG rickettsiae exhibiting *omp*A, respectively (Fournier et al. 2003). Based on this analysis, the agents can be clustered into several genetic lineages, providing the molecular parameters for defining new species within related group of isolates. Analysis of these gene fragments and determining their levels of genetic similarity is useful for distinguishing *Rickettsia* mostly on the species level and confirming their inclusion in the genus *Rickettsia*. These fragments also contain diagnostic restriction enzyme sites permitting a similar level of species identification without costly sequencing methods and allowing rapid screening of many samples in epidemiological investigations (Eremeeva et al. 1994; Roux et al. 1996). However, the relatively low level of discrimination afforded at the species level limits the usefulness of the targets for molecular epidemiology.

3.3 Quantitative PCR and Fluorescence-Based Detection Technology

Quantitative PCR assays take advantage of the whole genome sequences available for almost 100 isolates of *Rickettsia*. Genome analysis permits design of unique diagnostic assays to perform broad-range or species-specific detection and identification of rickettsial DNA both in clinical and environmental samples. As a rule, fluorescence-based detection allows for enhanced sensitivity compared to single-step traditional PCR methods. Various formats employed for these purposes include TaqMan (Eremeeva et al. 2012; Kato et al. 2013; Denison et al. 2014), SYBR Green assays which are currently improved by use of EvaFastGreen (Eremeeva et al. 2003a, b), and molecular beacon assays (Henry et al. 2007). Additional optimization in primer design such as inclusion of locked nucleic acid probes leads to further improvement of the specificity of the detection method, as was utilized in a duplex assay for simultaneous detection and identification of *R. typhi* and *R. felis glt*A fragment in investigations of flea-borne rickettsioses in California (Abramowicz et al. 2012; Eremeeva et al. 2012). Advantages of these advanced molecular methods have been demonstrated for differential diagnosis of murine typhus and spotted fever group rickettsioses in Tunisia (Khrouf et al. 2016). Improvements in molecular methods used for diagnosis of Japanese spotted fever facilitated acute stage patient management and ensured timely therapy of this potentially fatal illness (Kondo et al. 2015). The most recent approach to detection of *Rickettsia* and identification of cases due to RMSF,

developed by the Centers for Disease Control researchers, employs broad-range genus level identification of both typhus and spotted fever group rickettsiae followed by a confirmatory detection assay for *R. rickettsii* (Kato et al. 2013). Such an approach may be further developed for species-specific detection and identification of each of the species of *Rickettsia* as has been done for *R. parkeri* and *R. akari* (Denison et al. 2014). Similar species-specific assays using *omp*B were developed and applied for other *Rickettsia* species, both for case studies and for ecological assessments (Henry et al. 2007; Jiang et al. 2010, 2012).

3.4 Intergenic Regions, VNTR, INDEL, and SNP Typing

Analysis of the gene coding regions of *Rickettsia* indicated a high level of conservancy even between distant lineages of *Rickettsia*, and therefore these conserved sites are less useful for genotyping at the isolate level than intergenic regions (see Sect. 3.3) (Fournier et al. 2004; Fournier and Raoult 2007; Eremeeva and Dasch 2009). Therefore, more variable intergenic regions and other genomic loci containing tandem repeats, and sites exhibiting insertions or deletion events (INDEL) were evaluated in search of additional markers informative for species and strain identification. Diversity in single nucleotide polymorphisms, called SNPs, has also been examined by comparison of the whole genome sequences of several isolates of *R. rickettsii*, *R. typhi*, and *R. prowazekii* (Eremeeva and Dasch 2009; Kato et al. 2010; Bishop-Lilly et al. 2013; Clark et al. 2015).

3.4.1 Typing Based on Variations in Sequences of Intergenic Regions

Our study summarizing differences among the intergenic regions (IGR typing) found in 35 isolates of *R. rickettsii* of human, tick, and animal origin has been published in detail (Karpathy et al. 2007). Seven genotypes of *R. rickettsii* in four primary groups could be distinguished based on diversity in six variable sites. These separate groups include (1) isolates from Montana, (2) isolates associated with human infections and *Rh. sanguineus* ticks in Arizona, (3) isolates from the USA where *D. variabilis* is thought to be the primary vector, and (4) the isolates primarily associated with *Amblyomma* ticks from Central and South America. Inclusion of the lab attenuated Iowa isolate was based on *in silico* analysis. The latter was identical to six isolates including 84JG, Hino, OSU 83-13-4, OSU 84-21C, 76RC, and Hauke in these IGR sites; they all represent a slightly divergent group associated with *D. variabilis.* Isolate Hlp#2 is considered to be a non-pathogenic isolate of *R. rickettsii* in the USA (Parker et al. 1951, Paddock et al. 2014). It exhibited the most diversity from the other isolates of *R. rickettsii* examined and all differed significantly from another near relative, *R. philipii* 364D.

A similar IGR (multispacer typing-MST) approach was useful in determining the genetic heterogeneity of isolates belonging to *R. conorii* as it identified 27 different genotypes in the 39 *R. conorii* isolates examined (Fournier et al. 2004). It was also informative in demonstrating genetic diversity among *R. prowazekii* infecting human body lice during an outbreak of epidemic typhus in Burundi (Zhu et al.

2005), and provided additional molecular evidence to justify the subspecies description of *R. sibirica* subsp. *mongolitimonae* subsp. nov. (Fournier et al. 2006). In our hands IGR comparisons of isolates of *R. sibirica* did not exhibit any interspecies genetic variations, including isolates separated by spatial and temporal parameters based on documented passage history available for each isolate. In contrast we found genetic differences when we examined collections of *R. akari* isolates and *R. typhi* isolates (Kato et al. 2010; Tang et al. 2010). When applied on a global scale, MST combining the *dks*A-*xer*C, *mpp*A-*pur*C, and *rpm*E-tRNAfMet IGR regions identified 61 genotypes enabling differentiation of each of 23 valid *Rickettsia* species by at least one distinct site (Fournier and Raoult 2007).

3.4.2 Typing Based on Tandem Repeats

Tandem repeats (TR) are sites in the genome where an identical or nearly identical nucleotide sequence is present in two or more adjacent copies. They are related to minisatellites and consist of repeats of 10 to over 100 base pairs, while microsatellites are repeating sequences of 1–6 nucleotides. If the copy number of repeats differs between individual isolates they are referred to as variable numbers of tandem repeat sites (VNTR). When VNTR repeats are greater than 6 bp, they can be identified by low technology agarose gel electrophoresis rather than by DNA sequencing. Some characteristics of TR found in *R. rickettsii* Sheila Smith and their comparison to homologous elements present in other species of *Rickettsia* and *Orientia* were previously reported (Eremeeva and Dasch 2009). PCR amplicons of four out of 12 VNTR sites selected from the genome of *R. rickettsii* had variability in electrophoretic mobility using the same panel of isolates used previously for IGR typing. The analysis of their concatenated sequences identified 18 genotypes of *R. rickettsii* clustered into six groups. Like IGR typing this grouping also correlated with the specific tick vector from which the isolates were obtained or where these ticks are prevalent including human and animal isolates (Eremeeva and Dasch 2009). Furthermore, grouping of Iowa isolate was confirmed as typical of other *R. rickettsii* isolates associated with *D. variabilis* but it exhibited one new unique VNTR type due to gene RR1030 encoding for a hypothetical protein with ankyrin-like repeats. Two other VNTR sites exhibiting unique genotypes in Hlp#2 and *R. philipii* 364D isolates were identical in all other isolates of *R. rickettsii* including the Iowa isolate. The VNTR typing system was used in molecular epidemiology to demonstrate the unique lineage of *R. rickettsii* isolates associated with *Rh. sanguineus* in Arizona (Eremeeva et al. 2006a, b), and the distinctive genotype of *R. rickettsii* found in *Rh. sanguineus* in southern California (Wikswo et al. 2007). Furthermore, the *R. rickettsii* circulating in *Rh. sanguineus* in Mexicali, Mexico is a unique genetic type that is different from strains detected in Arizona and South and Central America (Eremeeva and Dasch 2009; Eremeeva et al. 2011). In contrast, it was established that *R. rickettsii* circulating in Panama share the same *ric*A VNTR genotype as isolates found in Central and Southern America and previously associated with *A. cajennense* (Estripeaut et al. 2007).

3.4.3 Typing Based on INDEL and SNP Sites

Analysis of the genome sequences of the *R. rickettsii* isolates Sheila Smith and Iowa identified 143 INDELs ranging in size from 1 to 10,585 bp (Ellison et al. 2008). Only 33 % of a total of 143 deletions identified were within predicted coding regions of *R. rickettsii,* of which 23 were in Sheila Smith isolate compared to Iowa, and 24 in Iowa compared to Sheila Smith. Further comparative whole genome analysis of several other isolates of *R. rickettsii* demonstrated that the eastern (Iowa and Morgan) and western strains (Sheila Smith and Bitterroot) clearly were distinguished, although there was approximately 99 % identity among the four strains of *R. rickettsii* (Clark et al. 2015). Only 12 non-synonymous SNPs (four in *ompA*), six synonymous SNPs, and eight SNPs in intergenic regions distinguished Sheila Smith from the Bitterroot (R) strain. Similarly, only eight unique non-synonymous SNPs specifically distinguished the Morgan and the Iowa strains. In addition, 8 synonymous SNPs and 15 SNPs in intergenic regions uniquely differentiated the Iowa and Morgan strains. Eight insertions/deletions, five in noncoding regions and three in coding regions, completed the distinctions between Iowa and Morgan.

More extended comparison of whole genome sequences of *R. rickettsii* representative of each genetic lineage (identified above) resulted in finding of eight informative sites which permitted identification of nine genetic clades within *R. rickettsii* (Kato et al. 2009). Whole genome sequence INDEL and SNP analysis were in agreement with previous IGR and VNTR analysis and corroborated its conclusions about unique positions for Hlp#2 and *R. philipii* 364D consistent with a distinct subspecies of *R. rickettsii* and a unique species of spotted fever group rickettsiae, respectively (Karpathy et al. 2007; Eremeeva and Dasch 2009).

INDEL site analysis provided additional genetic data to distinguish *R. prowazekii* and *R. typhi* (Dasch et al. 2006). One hundred and ten INDEL sites were analyzed by PCR with 38 isolates of *R. prowazekii* and 18 of *R. typhi*. Eight of these sites exhibited sequence polymorphisms, two that were unique to Madrid E and one for *R. typhi*, and these sites could be used to differentiate among flying squirrel isolates of *R. prowazekii*. Comparison of the genome sequence of the *A. cajennense* tick isolate from Mexico with the Madrid E sequence resulted in identification of several new INDELs unique to *R. prowazekii* (Zhu et al. 2008). Six variable loci were compared by DNA sequencing of eight isolates of *R. prowazekii* including Madrid E and EVir, two flying squirrel isolates, Breinl, and three isolates from Africa (ZRS, Cairo, and Addis Ababa). Three SNPs were present in the INDEL regions, and only ZRS and Addis Ababa isolates could not be distinguished among the seven genotypes identified.

Multi-locus genetic typing based on sequencing of small INDELs or SNPs is very informative but may be prohibitive due to the lack of sequencer access in the clinical or field settings. In contrast, sites containing tandem repeats or large INDELs may be the most useful for isolate typing since they can be detected without DNA sequencing in many cases as seen in an INDEL in the *glt*A coding region (Regnery et al. 1991; Eremeeva et al. 2003a, b), variations in numbers of tandem repeats of *omp*A detected in different isolates and spontaneous loss of repeat elements during in vitro passages of *R. rickettsii* isolate Sheila Smith (Gilmore and Hackstadt 1991; Matsumoto et al.

1996). Divergences in VNTR and INDEL sites in ORFs as well as in intergenic regions may or may not alter gene functions or gene regulation, respectively, and thus affect the virulence of individual isolates; however, these correlations are not yet studied in depth for *Rickettsia*. However, they provide powerful tools for distinguishing different isolates of individual species of *Rickettsia* for use in molecular epidemiology.

Availability of whole genome sequences for most known species of *Rickettsia*, as well as for humans and their arthropod vectors will open new opportunities for searching for informative and discriminating signature molecular markers to address many critical questions related to the occurrence, prevalence, morbidity, transmission, and evolution of rickettsial diseases and the rickettsiae themselves (Table 2.3).

Table 2.3 Application of molecular typing methods and their possible outcomes

Application	Specific questions addressed	Outcomes (examples)
Outbreak investigations and case study	To fulfill case definitions for confirmed cases	Demonstrating co-circulation of two pathogenic rickettsiae in a single outbreak site
	To determine the source of an outbreak and establish if the cases are part of the same outbreak	Defining the etiology of RMSF outbreaks in Arizona, US and Mexicali, MX
	To confirm or refute inferences regarding etiological events	Sylvatic typhus outbreak investigation in PA flying squirrels
	To identify clusters requiring further investigation	Canine *R. massiliae* in Los Angeles, California
Surveillance of rickettsial diseases	To distinguish between time-space clusters and sporadic cases of the same disease	
	To estimate prevalence of infection and observe trends over the time	Active surveillance of *R. philipii* cases in California
	To identify the origin of case clusters that need further investigation	Arizona or Mexico sites for RMSF
	To detect and identify emerging pathogens and follow emergence of new infections(s)	Atlantic rain forest *Rickettsia* in South America, *R. philipii* cases in California
	To determine distribution of virulent strains	Better understanding of the ecology of RMSF
Understanding transmission and natural maintenance of rickettsial diseases	To determine the incidence and prevalence of occurrences of infections in the human, animal and arthropod populations	Improved One Health responses to disease
	To understand the natural history of infection and define the duration of infection in different hosts	Improved seasonality and site risk assessments for rickettsioses
	To decipher epidemiological parameters of agent and host interactions affecting the risk of transmission	Facilitation of public health control responses to vector-borne diseases

Broad acceptance of the utility of such markers and associated typing techniques will require their extensive evaluation and validation and practical experience with appropriate samples from epidemiological investigations. The sections below will review the challenges entailed in obtaining the types of materials needed for conducting molecular epidemiological investigations using the new tools.

3.5 Collection of Samples Needed for Molecular Epidemiological Investigations

Molecular detection and identification of rickettsiae in a clinical specimen from a patient with compatible acute symptoms is the most definitive method for confirmatory diagnosis of rickettsiosis (Biggs et al. 2016). A skin biopsy collected from the areas affected by rash and eschar are most suitable samples for these procedures for many rickettsioses because they contain more organisms than in blood samples; however, use of invasive procedures (almost a mini-surgery) for their collection makes them less than attractive for epidemiological studies. Consequently, an alternative method was sought, based on swabbing the scab area under an eschar and/or collecting the eschar itself followed by DNA extraction and molecular testing. This approach was first applied for detection and identification of *R. conorii* infection (Mouffok et al. 2011). It was also proven to be very productive for differential diagnosis and identification of rickettsial infection due to *R. philipii, R. parkeri,* and other SFG rickettsioses manifested with an eschar (Johnston et al. 2013; Myers et al. 2013; Portillo et al. 2013; Khrouf et al. 2016). It is expected that this approach for collecting diagnostic specimens will be more frequently used in clinical practice as it becomes clear that definitive diagnostic data can be obtained. Surveillance data obtained from such analyses will be quickly used by the medical community and public health professionals involved in epidemiology.

Archival pathology samples suitable for molecular testing and retrospective epidemiological analysis permits one to confirm the true rickettsial cases in patients with similar clinical manifestations and to identify new etiologies. Formaldehyde-fixed and paraffin-embedded (FFPE) tissue is a very good source of material for retrospective diagnosis of rickettsioses using antibody-based immunohistochemical staining methods but more difficult to use reliably for molecular analyses. As previously mentioned the immunohistochemical methods are similar to other serological methods in not providing the exact etiological identification unless species-specific monoclonal antibodies are utilized (Paddock and Eremeeva 2007; Paddock et al. 2008). Many studies have used DNA extracted from de-paraffinized tissues for complete investigation of fatal cases (Rozental et al. 2006) and conduct retrospective analysis of cold cases (Denison et al. 2014; Paddock et al. 2014). It is likely that new extraction and repair methods coupled with more sensitive next generation sequencing will allow a larger portion of FFPE samples to yield definitive diagnoses. Use of only fixed tissues permits timely and accurate diagnosis of disease(s)

and proper case management and public health responses without unnecessary alarms, especially in the context of differential diagnosis of another possible febrile illness with cutaneous manifestations such as anthrax. As an example, a cluster outbreak due to *Rickettsia akari* in NY city residents was originally associated with and attributed to exposure to *B. anthracis* spores (Paddock et al. 2003). Similarly, a suspicion of cutaneous anthrax in an elderly resident of California led to identification of the new rickettsial pathogen, *R. philipii,* whose potential for causing human illness was long suspected but could not be determined due to the lack of an adequate clinical sample (Lane et al. 1981a, b; Shapiro et al. 2010). Once confirmed, recognition of *R. philipii* as human pathogen changed the focus of rickettsial disease surveillance in California leading to identification of new cases, expanding the range of rickettsial disease expected across the state and to a better understanding of the clinical manifestations of this disease in pediatric and adult patients (Shapiro et al. 2010; Johnston et al. 2013; Padgett et al. 2016).

These examples and others below indicate that epidemiological studies will need better and more reliable samples to permit a routine and systematic molecular approach to surveillance of rickettsial diseases which is based on use of the most efficient laboratory diagnostic tools and the most informative specimen types. This will require looking beyond collection of traditional samples and evaluation of proxy case-associated sources including animals and ectoparasites in the vicinities of the human cases which may provide invaluable clues for more complete and accurate purpose of public health surveillance. This in turn will lead to better education of physicians about the epidemiological clues to record and for the public to better assess the risk of rickettsioses to their health.

4 Applications of Molecular Epidemiology in Outbreak Investigations of Rickettsial Diseases

4.1 Molecular Epidemiology of the Emerging Rickettsiosis due to Rickettsia massiliae

Parola et al. reported co-circulation of *R. conorii*, the agent of Mediterranean spotted fever, and the emerging pathogen *R. massiliae* in a focus of spotted fever in France in May 2007 (Parola et al. 2008). Only serological tests were available for the patients affected, and the differential diagnosis was established by serum cross-adsorption and western blotting rather than by molecular methods. However, ecological assessment and tick testing contributed molecular evidence for the presence of *R. massiliae* in areas traditionally thought to be endemic only for Mediterranean spotted fever. The frequent detection of *R. massiliae* in *Rhipicepalus* ticks in different geographic locations indicates that this ubiquitous emerging pathogen may contribute to yet an unknown portion of Mediterranean spotted fever and may be an emerging rickettsiosis in both North and South America and

should be considered as a part of every differential clinical diagnosis (Zaharia et al. 2016). This is to ensure proper patient management and to avoid unexpected antibiotic treatment failures since *R. massiliae* is known for its resistance to rifampin (Rolain et al. 1998).

While the presence of *R. massiliae* in the USA is confirmed by detection and isolation of this agent from brown dog ticks (Eremeeva et al. 2006a, b; Beeler et al. 2011; Fornadel et al. 2013), no human cases have been yet detected in the USA. This agent is associated with severe hemorrhagic presentations or causes neck lymphadenopathy in patients from South America and Mediterranean region (Garcia-Garcia et al. 2010; Zaharia et al. 2016). Probable cases of canine rickettsiosis due to *R. massiliae* were reported in Los Angeles (Beeler et al. 2011), thus implying that similar human causes are likely being overlooked (Demma et al. 2006), or different virulence properties are exhibited in the US *R. massiliae* compared to the South American and European isolates.

4.2 Molecular Epidemiology of RMSF

The significant contribution of molecular tools to confirming and investigating cases of RMSF in atypical ecological settings is a powerful example of the utility and epidemiological impact of this new discipline. In the now classic example of the emerging focus of RMSF in arid Arizona (Demma et al. 2005), it was originally thought to be a mystery illness with fatal complications in both pediatric and adult patients. RMSF was unexpected there due to the lack of a typical *Dermacentor* vector for RMSF and the lack of host wild animal associations, unusual climate, and strong misconceptions related to the vectorial capacity of American *Rh. sanguineus*. Preliminary molecular findings backed up by isolation of *R. rickettsii* from the brown dog ticks and clinical samples collected in Arizona refuted those outdated beliefs (Demma et al. 2005; Eremeeva et al. 2006a, b), and led to a revised understanding of the eco-epidemiology of RMSF in the USA. The epidemiology was crystal clear: a novel genotype of *R. rickettsii* was found in Arizona and identified in both patients and *Rh. sanguineus* from sites with human infections (Eremeeva et al. 2006a, b; Karpathy et al. 2007). Subsequent outbreaks with similar epidemiological and ecological attributes occurred a few years later in Mexicali, Mexico (Tinoco-Gracia et al. 2009; Alvarez Hernández and Contreras Soto 2013), providing grounds for speculation about possible common sources of both outbreaks due to their close geographic proximity, common presence of dogs and brown dog ticks and absence of other tick vectors for *R. rickettsii*. Here again, molecular tools provided objective evidence for independent origins of RMSF in Mexicali and Arizona based on the differing genetic types of *R. rickettsii* and *Rh. sanguineus* present in each locale (Eremeeva et al. 2011). These findings excluded exchange of ticks and agents between two sites as an imported origin of infections and they are thus autochthonous. Interestingly, the potential importation of Mexican brown dog ticks to the USA as a source of the outbreak had been highly anticipated. This speculation

instigated an intensive active environmental investigation focused on California border animal shelters and roaming dogs; however, no molecular evidence was found that Mexicali was the recent source of any rickettsial disease in the USA (Fritz et al. 2012; Eremeeva, personal communication) or conversely that the disease in Mexico had been imported from Arizona. Much remains to be done as numerous other sites with RMSF and *Rh. sanguineus* have now been identified in Mexico and it is suspected in Brazil (Szabo et al. 2013a, b). The genotypes of these additional reservoirs of *R. rickettsii* have not yet been reported.

4.3 Molecular Epidemiology of Rickettsioses due to R. parkeri *and Atlantic Rainforest* Rickettsia *sp.*

While *R. parkeri* was thought to be the sole cause of a spotted fever rickettsiosis with eschar in both North and South America since it was first reported in 2004, identification of the Atlantic rainforest *Rickettsia* sp. (ATRF) demonstrated the great importance of molecular epidemiology in disproving that idea. Two clinical cases were independently identified by two research groups working on diagnosis of an eschar-associated rickettsial illness in Brazilian patients both with histories of outdoor activities (thus exposure), one in Bahia and the other in Sao Paulo (Spolidorio et al. 2010; Silva et al. 2011). These two small case reports lead to extensive studies that resulted in isolation of this novel *Rickettsia* sp. and a better understanding of the ecology and epidemiology of this *Rickettsia* as well as the risk factors for potential exposure to and infection with this pathogen (Szabo et al. 2013a, b). It led to efforts demonstrating that disease caused by this agent extended beyond Brazil into Colombia and Argentina (Londono et al. 2014; Monje et al. 2015). ATRF is so closely related to *R. parkeri* that it was first thought to be a strain or geographic variant (Spolidorio et al. 2010). Both organisms belong to the same genetic lineage as *R. africae* and *R. sibirica*; phenotypic and some genetic similarities between *R. parkeri* and *R. africae* caused a debate whether they all represented geographic variants of the same single species of spotted fever group rickettsiae (Goddard 2009). However, it is now clear that ATRF and *R. parkeri* represent two different rickettsial pathogens each with their own ecology and tick vectors. Atlantic rainforest *Rickettsia* is transmitted by *Amblyomma ovale*. Neither agent is closely related to *R. africae* at the complete genome sequence level of analysis.

The clinical features of infection caused by *R. parkeri* are generally less severe than those produced by *R. rickettsii*, and its hallmark is that patients have at least one eschar (Paddock et al. 2008) while these are rare or do not occur with *R. rickettsii*. The etiological significance of *R. parkeri* was established in 2004 after its DNA was PCR amplified from the eschar tissue of a patient from southeast Virginia followed by its culture isolation (Paddock et al. 2004). Subsequently, the geography of US human cases was expanded from Texas to Maryland and Carolinas to Kansas and Oklahoma where it is thought to be primarily transmitted by the Gulf tick, *Amblyomma maculatum* (Paddock et al. 2008); the most recent case due to *R.*

parkeri was reported from Arizona (Herrick et al. 2016). *A. maculatum* is also found in the Caribbean and northern South America. Outside of the USA, clinical cases of *R. parkeri* infection are reported from Argentina and Uruguay (Romer et al. 2011, 2014; Venzal et al. 2012; Portillo et al. 2013). *Rickettsia* parkeri is primarily transmitted in South America by *A. triste, A. tigrinum, A. dubitatum*, and *A. nodosum* (Melo et al. 2015), and a closely related organism was found infecting *Amblyomma parvitarsum* in Chile and Argentina (Ogrzewalska et al. 2016).

4.4 The Challenges for Molecular Epidemiology in Identifying New Pathogens and Confirming Etiologies

The currently used case definition for rickettioses recognizes the confounding antigenic cross-reactivity among known pathogenic *Rickettsia* and relies on availability of molecular data to determine their identity. This is of particular importance because as of today over 20 species of spotted fever group rickettsiae are recognized as human pathogens and many other novel rickettsial species with no known associations with human diseases are discovered worldwide (Parola et al. 2013). Among those, three tick-borne rickettsioses are known to be endemic to the USA — Rocky Mountain spotted fever caused by *R. rickettsii*, *R. parkeri* rickettsiosis, and Pacific Coast Tick spotted fever caused by *R. philipii*. Furthermore, it is speculated that *R. amblyommii*, *R. andeanae*, and *R. montanensis* may contribute to some illness or repeated exposures causing formation of anti-rickettsial antibodies and seroconversion (Billeter et al. 2007; Apperson et al. 2008; McQuiston et al. 2012; Paddock et al. 2015). However, there are no laboratory confirmed cases with direct molecular evidence for infection with those agents. Speculations have been associated for many years with *R. amblyommii*, one of the most frequently detected SFGR which infects the aggressive human biting Lone Star tick, *Amblyomma americanum* (Stromdahl et al. 2011). It has been recently demonstrated that *R. amblyommii* is the most probable *Rickettsia* infecting people in high RMSF reporting areas but where *R. rickettsii* is rarely found in ticks (Moncayo et al. 2010; Delisle et al. 2016).

The frequent exposure of people to the flea-borne *R. felis* group of agents and sporadic exposure to mite-transmitted *R. akari* is likely widespread but often goes unnoticed due to the asymptomatic or very mild manifestations of associated diseases in overall healthy populations (Paddock and Eremeeva 2007; Civen and Ngo 2008). The pathogenic potential of *R. felis* is not yet fully understood despite its almost ubiquitous presence in flea samples (Reif and Macaluso 2009); moreover, recent discoveries of closely related flea-borne relatives, namely *Candidatus* R. senegalensis and *Candidatus* R. aesemboensis, and other genetic variants more closely related to the type strain of *R. felis* in the US and abroad requires better appreciation of the potential biological and pathogenic complexity of this group of agents and flea endosymbionts (Jiang et al. 2013; Mediannikov et al. 2015;

Angelakis et al. 2016). Both *Candidatus* R. senegalensis and *Candidatus* R. aesemboensis are known from the USA (Billeter et al. 2016). While application of species specific molecular detection and identification tools should allow further insight into this problem, especially in the endemic areas where *R. typhi* is also prevalent, it will require collection of appropriate clinical specimens to prove the human pathogenicity of each agent. Since no human or veterinary clinical isolates have been made in the USA and *R. felis* can be difficult to cultivate, this remains a significant challenge.

5 Application of Molecular Tools for Surveillance of Rickettsial Diseases

The potential for the contribution of molecular epidemiological tools and principles to the routine surveillance of rickettsial diseases is great. A long list of current epidemiological questions is related to our incapacity for differentiating between causes of temporal and spatial clusters and sporadic cases of the same rickettsiosis occurring at different geographic locations and times. We need to recognize new and atypical clusters requiring further investigations as they may involve detection and characterization of new pathogens, and their relative contributions to accurate estimations of infection and forecasting trends. It is believed that the current prevalence of even classic rickettsial diseases is largely underreported due to confusion with nonspecific flu-like symptoms, and the noncontributory role of negative laboratory test results during the acute stage of illness or infections with low or negligible antibody responses with current antigens and tests (Biggs et al. 2016). Focusing on improved molecular approaches can provide the missing information needed to define the actual prevalence of these illnesses and associated extents of human exposures. These efforts will be necessary to better understand the true societal burden and costs of these diseases and their long-term outcomes on patient health (Archibald and Sexton 1995; Bergeron et al. 1997; Carr et al. 2014). Unfortunately, many of these approaches are difficult to implement due to the limited real-time collection of appropriate clinical samples and the sporadic and inefficient detection of cases suitable for such sampling due to the lags inherent in the passive surveillance reporting system used in the USA and most other countries. However, a few active surveillance efforts have clearly demonstrated the advantages and limitations of using molecular platforms in such investigations.

RMSF is the most commonly diagnosed rickettsiosis in Tennessee; this state is also one of those reporting the greatest number of cases and associated mortality (Adjemian et al. 2009). Given these results from the reporting system, it is notable that extended environmental surveillance projects failed to identify *R. rickettsii* in any ticks collected across Tennessee (Moncayo et al. 2010). In contrast, cross-adsorption experiments were performed using sera of TN residents found to be IFA positive for RMSF based on commercial testing. Accordingly, 55.4 % ($N = 31$) had specific reactivity to *R. amblyommii* rather than *R. rickettsii* (Delisle et al. 2016).

The same individuals experienced fever (75 %), headache (68 %), and myalgia (58 %), but rash (36 %) and thrombocytopenia (40 %) were less common. These findings suggested an etiological role for *R. amblyommii* in these areas; previously some recognized cases had been attributed to *R. parkeri* and potentially to *R. montanensis* but these are relatively infrequent infections. Molecular data will be necessary to obtain final confirmation of these findings and inclusion of *R. ammblyommii* on the list of pathogenic rickettsiae. Obtaining these confirmatory data is critical, because this *Rickettsia* is harbored by an aggressive human biting tick in much of the eastern USA and depending on the geographic area, its prevalence is an average 41 % in questing ticks and ranges from 66.5 % to 80.5 % in ticks attached to humans (Mixson et al. 2006; Jiang et al. 2010), greatly exceeding the natural prevalence of any other spotted fever group rickettsiae, including *R. rickettsii* and *R. parkeri* (Sumner et al. 2007; Stromdahl et al. 2011).

Flea-borne rickettsioses in southern California are another disease needing better human molecular epidemiology. California is one of the US states where murine typhus due to *R. typhi* is a reportable disease, and where outbreaks have been reported for several continuous years (Civen and Ngo 2008). Detailed investigations involving PCR testing of thousands of fleas as well as animal tissues demonstrated that spatial differences are present in the occurrence of rickettsial agents (Abramowicz et al. 2011, 2012; Eremeeva et al. 2012). The classic urban murine cycle consists of rats and rat fleas (*Xenopsylla cheopis*) in Los Angeles; as expected they are associated with *R. typhi* there (Abramowicz et al. 2011), and with reports of murine typhus in humans. Similarly, *R. typhi* was found in the vicinity of suburban cases of murine typhus; however, its circulation appeared to be associated with cats, opossums, and cat fleas (Eremeeva et al. 2012). However, no evidence for the presence of *R. typhi* was detected in similar ecological settings where only opossums were captured and they all were infested only with cat fleas (Abramowicz et al. 2012). Cat fleas from both suburban environments were largely infected with *R. felis* which is reported worldwide as a human pathogen but only reported once in a patient case in the USA from Corpus Christi, TX (Schriefer et al. 1994). Consequently, it has been suggested that cat flea rickettsiosis due to *R. felis* is probably underdiagnosed by the medical community in the USA (Eremeeva et al. 2012). Most recent molecular information regarding the diversity of spotted fever group rickettsiae infecting cat fleas suggests that different genotypes also may have different pathogenic potential (Jiang et al. 2013; Mediannikov et al. 2015; Billeter et al. 2016), thus offering at least a partial explanation to the absence of clinical cases of cat flea rickettsioses in the USA since it was first discovered in Texas. Dynamics of the interactions of these two endemic cycles and the current status of the classic rat/rat flea cycle needed to maintain *R. typhi* are not well understood; however, it has been suggested that rat eradication programs may have led to host and vector change for murine typhus (Civen and Ngo 2008). Some observations in support of this hypothesis have been accumulated in Texas (Blanton et al. 2015a, b); however, further studies are needed to dissect these ecological interactions. Molecular confirmation of morbidity associated with each species of flea-borne rickettsiae will require evidence of *R. typhi* and other *Rickettsia* agents in clinical samples taken from patients with compatible symptoms and exposure history.

6 Use of Biomarkers of Rickettsial Infection and Future Directions for Molecular Epidemiology

Continued development of new molecular tools for detection and characterization of rickettsial agents is essential for all aspects of public health research relevant to these agents. This extends from outbreak investigations and routine surveillance to monitoring the course and outcomes of clinical illness, to understanding the dynamics of host–pathogen interactions, and to investigating factors affecting the environmental maintenance and transmission of rickettsiae. At present only a small number of molecules are targeted to obtain the confirmatory evidence for an acute rickettsial infection, but even fewer of them have ever been evaluated as reliable and informative markers in delayed illness (antibiotic, low-dose exposure, palliative or partially effective treatments, strong innate immunity) or at different stages of convalescent responses to rickettsial infection. Several studies have been conducted to determine the sensitivity of various DNA and antibody detection assays (Philip et al. 1977; Newhouse et al. 1979; Clements et al. 1983; Kato et al. 2013); however, these studies did not really address the issues related to the mechanisms of rickettsial interactions with vertebrate hosts, changes in class and affinity or specificity of antibody, as well as the presence and quantity of viable vs. non-viable rickettsiae in the bloodstream or infected sites of infected individuals. Similarly, only a few studies have been conducted to determine changes in cytokine profiles that occurred during the course of infection or to follow the metabolome during the course of disease (Damas et al. 2009; Forte et al. 2009; Popivanova et al. 2011; Bhavnani et al. 2013; Tai et al. 2014).

It is commonly accepted that rickettsioses occur following exposure to an infected arthropod, self-inoculation or in rare cases, aerosol exposure of airborne rickettsiae. Acute illness is typically characterized by a time-limited presence of rickettsiae in the patient blood stream due to the higher affinity of microorganisms to the endothelium followed by antibody responses with almost simultaneous rise in IgM and IgG class antibody titers starting at ~6–10 days of acute illness. The antibody titers continue to mount through the recovery period and circulate sometime for several years following this convalescent period. Inoculation of rickettsiae is also followed by specific pathological changes and release of numerous proinflammatory cytokines and other cellular factors. The details of these responses are covered in several outstanding reviews (Sahni and Rydkina 2009; Mansueto et al. 2012; Sahni et al. 2013). The utility of these changes as biomarkers of disease and their correlation with the severity of rickettsial infections, stage specific responses and association with particular agents needs further investigation before their value in molecular epidemiology can be assessed. Longitudinal gene expression (transcriptome) studies of patients suffering and recovering from rickettsioses may be very informative for addressing some of these questions; this type of study has identified differential gene expression signatures that occur during acute Lyme disease (Bouquet et al. 2016) and other diseases (Vigil et al. 2011).

Consequently, the greatest need in molecular epidemiology remains the development of more sensitive and specific serological diagnostic assays based on species and genus-specific markers; this is abundantly clear because serum remains the easiest and safest sample to obtain from suspect patients. However, this approach will never have the fine resolution of molecular screens at the single nucleotide level. Significant limitations inherent in current approaches to the diagnosis and surveillance of rickettsial diseases are also due to the very small collections of clinical samples suitable for testing that can be obtained. Some earlier reports indicated finding rickettsial antigen in urine (Fleck 1947; Fleck et al. 1960), but this may only occur during fulminant infections accompanied by severe damage to internal tissues with massive destruction of endothelium and shedding of rickettsiae from the damaged tissues. Alternative suitable samples for acute stage diagnosis such as cutaneous scab swabs have proven to be very effective and more acceptable compared to the more invasive collection of biopsies; however, this is applicable only to infections associated with eschar development (Mouffok et al. 2011; Johnston et al. 2013; Khrouf et al. 2016) unless a suitable equivalent method based on sampling rashes can be developed. Thus, concerted and costly efforts will be required to obtain better prospective samples useful for diagnosis and surveillance of rickettsial infections. Sampling of cell-free DNA (liquid biopsy) from blood coupled with sensitive next-generation sequencing may be one such approach. PCR methods coupled with sequencing or restriction fragment polymorphism analysis provide the most reliable identification of etiological agent; however, even in precisely choreographed and focused studies, the sensitivity of these methods remains very low. Consequently, new-generation detection platforms will require inclusion of various rickettsia enrichment and host material depletion procedures to ensure desirable levels of detection or detection at the single molecule level will be needed.

The initial targeted sequencing of selected genes from many rickettsial isolates was the critical first step in developing molecular diagnostic tools, and application of many of them has been discussed in previous sections. Some progress has been made in mining complete genome sequences of *Rickettsia* to design various species-specific and generic assays (Jiang et al. 2010, 2012, 2013; Kato et al. 2013; Denison et al. 2014). These have already resulted in significant improvement in the diagnostic capacity of many clinical laboratories (Kondo et al. 2015; Khrouf et al. 2016). The next step will require coupling of this information with identification of signature markers indicative of various stages of infections that are informative in their application to clinical diagnosis and population surveillance projects. Rickettsial infections are often under-recognized due to the commonality of early onset symptoms so they are misdiagnosed as dengue, malaria, or chikungunya (Zavala-Velazquez et al. 1996; Premaratna et al. 2011; Mediannikov et al. 2013). Furthermore, the patients may present with atypical exposure history, mixed infections, or unusual clinical manifestations hence complicating differential diagnosis and choice of treatment (Goncalves da Costa et al. 2002; Chaudhry and Scofield 2013; Blanton et al. 2015a, b). The beneficial impact of deep sequencing technology for clinical applications has been already demonstrated for identification of novel viruses and

other rare agents (Wilson et al. 2015). Applying similar approaches for rickettsial diseases may be promising as they may be used with *Rickettsia*-rich biopsy tissues and are proven to be the most informative and inexpensive method as costs for molecular methods continue to drop.

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Chapter 3
Biodiversity of "Non-model" *Rickettsiales* and Their Association with Aquatic Organisms

Michele Castelli, Davide Sassera, and Giulio Petroni

1 Introduction to the Currently Known Diversity of *Rickettsiales*

Rickettsiales are an order of obligate intracellular bacteria inhabiting eukaryotes (i.e., they cannot proliferate in host cell-free media), belonging to the *Alphaproteobacteria* class (Dumler and Walker 2005). For a long time, knowledge on *Rickettsiales* was restricted almost exclusively to its pathogenic and medically relevant members belonging to the genera *Rickettsia*, *Anaplasma*, *Ehrlichia*, and to reproductive manipulators in insects of the genus *Wolbachia* (Dumler et al. 2001; Dumler and Walker 2005).

In the past few decades, thanks to molecular and phylogenetic data, the view on the evolutionary relationships within *Rickettsiales* significantly changed. The revision operated by Dumler and coauthors in 2001 (Dumler et al. 2001) reorganized the taxonomy of *Rickettsiales* and their two "traditional" families *Rickettsiaceae* and *Anaplasmataceae*, which thereafter included, respectively, the two genera *Rickettsia*, *Orientia*, and the four genera *Anaplasma*, *Ehrlichia*, *Wolbachia*, and *Neorickettsia*. In the same years, it was already becoming evident that representatives of those known genera, such as *Rickettsia* and *Wolbachia*, displayed a broader host range than previously recognized, being retrieved, respectively, in several non-hematophagous arthropods (Werren et al. 1994; Chen et al. 1996; reviewed in Perlman et al. 2006; Weinert et al. 2009), and in filarial nematodes (Sironi et al.

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1995; Bandi et al. 1998; reviewed in Taylor et al. 2005; Werren et al. 2008). More recently, a much wider diversity became evident for *Rickettsiales*, both in terms of number of their representatives and of the eukaryotic hosts to which they were found associated. Indeed, completely new genus-level (e.g., Friedman et al. 2000; Sassera et al. 2006; Schrallhammer et al. 2013; Mediannikov et al. 2014) and even family-level (Montagna et al. 2013) clades of *Rickettsiales* were discovered.

Some findings in particular led to a prominent reshaping of the present views on the diversity, evolutionary, history, and host adaptation of *Rickettsiales*:

- Several new genus-level clades were identified within the two known families, resulting in six new genera of *Rickettsiaceae* and three new genera of *Anaplasmataceae* (Fig. 3.1; Table 3.1).
- A new family level lineage was discovered, which recently received a formal taxonomic status as "***Candidatus* Midichloriaceae**" (Montagna et al. 2013). Its diversity and host ranges are at least comparable to the other two families, with nine genera presently recognized (Fig. 3.1; Table 3.1).
- Out of the 18 total new genera of *Rickettsiales*, 14 were retrieved exclusively in association with organisms living in aquatic environments. Additionally, some representatives of two out of the six already recognized genera were as well found associated to aquatic organisms. Therefore, it results that the majority of presently known *Rickettsiales* genera are hosted by aquatic organisms, in several cases protists. Considering also the phylogenetic relationships (Fig. 3.1), these findings provide a strong indication that ancestral hosts of *Rickettsiales* were likely aquatic eukaryotes, and that the adaptation to arthropod hosts occurred many times independently along their evolutionary history, namely at least twice for each of the three families (*Rickettsiaceae*, *Anaplasmataceae*, "*Candidatus* Midichloriaceae") (Fig. 3.1).

Fig. 3.1 (continued) (values below 70 % were omitted). The trapezoidal or triangular shapes represent collapsed groups of closely related organisms. Organisms (or groups of organisms) which are at least in some cases associated to aquatic environments are shown in blue, otherwise, if associated only to terrestrial environments, in green. The inferred ancestors are shown as well in blue or green, if all known descendants were retrieved from the same kind of environment. On the right side, drawings exemplifying the typical host organisms, if known, are shown. The tick shape stands for "hematophagous arthropods," the bug for "non-hematophagous arthropods," the human for "humans and terrestrial vertebrates," the coral for "aquatic invertebrate animals (including corals, hydra, placozoans, mollusks, leeches, trematodes, ascidians, and echinoderms)," the amoeba for "amoeboid protists (including Acanthamoeba, nucleariids and rhizarians)," the flagellate for "flagellate protists (including chlorophytes and euglenozoans)," the roundworm for "parasitic nematodes," the fish for "fish." Rickettsiales that are associated to protists are shown in bold. For more details on the Rickettsiales symbionts and their hosts, see the text. For reference, NCBI accession numbers are provided for the sequences of uncultured bacteria that are not described in the text. Ca. is an abbreviation for Candidatus. The bar on the bottom stands for 10 % estimated sequence divergence

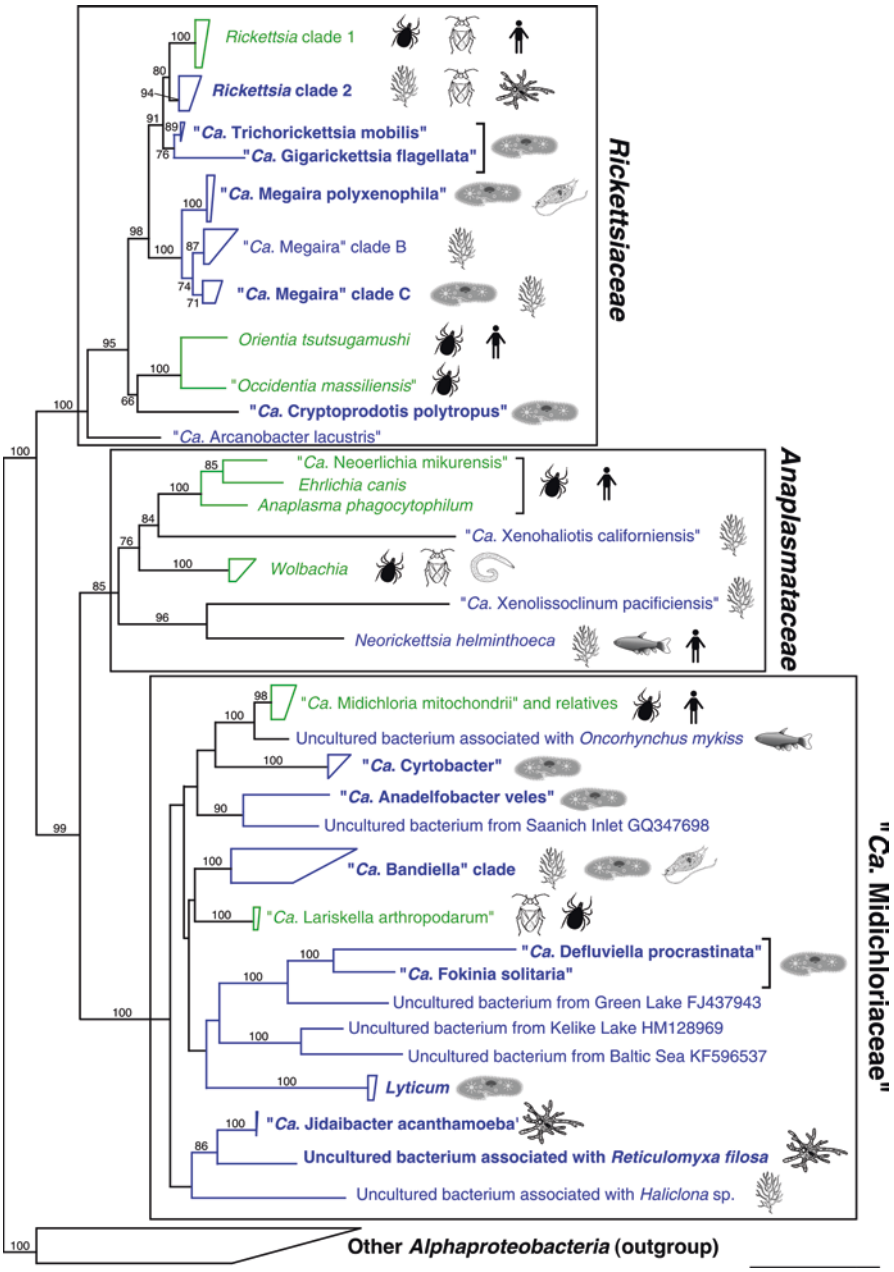

Fig. 3.1 Phylogenetic tree showing the evolutionary relationships among the main lineages within the three families (*Rickettsiaceae*, *Anaplasmataceae*, and "*Ca*. Midichloriaceae") of *Rickettsiales*. The 75 employed sequences were aligned using the software package ARB (Ludwig et al. 2004), and removing positions with gaps, obtaining 1172 nucleotides columns. The tree was inferred with a maximum likelihood approach employing the software PhyML (Guindon and Gascuel 2003). Numbers on branches refer to bootstrap percent support after 1000 pseudo-replicates

Table 3.1 List of new *Rickettsiales* genera discovered after the revision by Dumler and coauthors (Dumler et al. 2001)

Family	Genus	Host	Flagella	Genome data	Original molecular characterization and/or description
Rickettsiaceae	"***Ca.* Trichorickettsia**"	**Ciliate protists**	**Yes (in some hosts)**	**No**	Vannini et al. 2014
	" *Ca* . Gigarickettsia "	***Spirostomum minus* (ciliate)**	**Yes**	**No**	Vannini et al. 2014
	" *Ca* . Megaira "	**Ciliates , chlorophytes , streptophytes , hydra, corals, etc**	**No**	**No**	Vannini et al. 2005; Schrallhammer et al. 2013
	" *Ca* . Cryptoprodotis "	***Pseudomicrothorax dubius* (ciliate)**	**No**	**No**	Ferrantini et al. 2009
	"*Occidentia*"	*Ornithodoros sonrai* (tick)	Yes	Yes	Mediannikov et al. 2014
	" *Ca* . Arcanobacter"	**Not known**	**Yes (genes)**	**Partial**	Martijn et al. 2015
Anaplasmataceae	"*Ca.* Neoehrlichia"	Ticks, rodents	No	No	Kawahara et al. 2004
	" *Ca* . Xenohaliotis"	***Haliotis* spp. (gastropod mollusks)**	**No**	**No**	Friedman et al. 2000
	" *Ca* . Xenolissoclinum"	***Lissoclinum patella* (ascidian)**	**Yes (genes)**	**Yes**	Kwan and Schmidt 2013
" *Ca* . Midichloriaceae"	"*Ca.* Midichloria"	Ticks	Yes (genes)	Yes	Beninati et al. 2004; Sassera et al. 2006
	"*Ca.* Lariskella"	Arthropods	No	No	Mediannikov et al. 2004; Matsuura et al. 2012a
	" *Ca* . Anadelfobacter "	***Euplotes harpa* (ciliate)**	**No**	**No**	Vannini et al. 2010
	" *Ca* . Cyrtobacter "	***Euplotes* spp. (ciliate)**	**No**	**No**	Vannini et al. 2010
	" *Ca* . Defluviella "	***Paramecium nephridiatum* (ciliate)**	**No**	**No**	Boscaro et al. 2013b
	Lyticum	***Paramecium* spp . (ciliates)**	**Yes**	**No**	Boscaro et al. 2013a; Preer et al. 1974; Preer and Preer 1982

Family	Genus	Host	Flagella	Genome data	Original molecular characterization and/or description
	"*Ca*. Bandiella"	**Ciliates, euglenids, hydras, corals**	**Yes (genes)**	**Partial**	Fraune and Bosch 2007; Senra et al. 2016
	"*Ca*. Jidaibacter"	***Acanthamoeba* sp. (amoeba)**	**Yes (genes)**	**yes**	Fritsche et al. 1999; Schulz et al. 2015
	"*Ca*. Fokinia"	***Paramecium* sp. (ciliate)**	**No**	**No**	Szokoli et al. 2016

Organisms retrieved from aquatic environments are reported in bold. Protist hosts, as well as the genera that were retrieved in association with such organisms are underlined

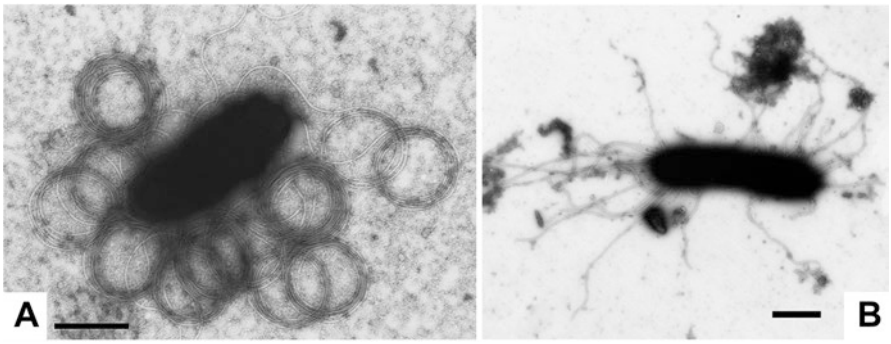

Fig. 3.2 Negative staining transmission electron microscopy images of *Rickettsiales* endosymbionts bearing flagella. (**a**) "*Candidatus* Trichorickettsia mobilis" from *P. multimicronucleatum* LSA: numerous and long flagella are clearly visible. Bar: 0.5 μm. (Vannini et al. 2014, doi:10.1371/journal.pone.0087718.g001). (**b**) *Lyticum flagellatum* harbored by *P. octaurelia* strain 299 showing several peritrichous flagella. Bar: 1 μm (Boscaro et al. 2013a, doi:10.1038/srep03305)

- The newly discovered lineages, in particular those associated to aquatic organisms, presented distinctive and unforeseen features, such as flagella or flagellar genes (Fig. 3.2), retrieved in all the families ("***Ca***. **Trichorickettsia,**" "***Ca***. **Gigarickettsia,**" "***Ca***. **Arcanobacter**" among *Rickettsiaceae*, "***Ca***. **Xenolissoclinum**" among *Anaplasmataceae*, "***Ca***. **Midichloria,**" ***Lyticum***, "***Ca***. **Jidaibacter,**" "***Ca***. **Bandiella**" among "*Ca*. Midichloriaceae"), bacterial chemotaxis genes ("***Ca***. **Arcanobacter**" among *Rickettsiaceae*), and nuclear localization in the host cells ("***Ca***. **Trichorickettsia,**" "***Ca***. **Megaira**" among *Rickettsiaceae*).

The study of the medically relevant *Rickettsiales*, especially when a comparison among these pathogens is made, should therefore properly take into comprehensive account their evolutionary history, including the phylogenetic relationships with the nonpathogenic *Rickettsiales* associated with aquatic organisms. Such an approach has the potential to offer a better understanding of the features of the well-known *Rickettsiales*, including those involved in pathogenesis. Considering the above-exposed premises, this chapter will present in an evolutionary framework:

- Those ***Rickettsiaceae*** and ***Anaplasmataceae*** found in association with **aquatic organisms**.
- The new *Rickettsiales* family "***Candidatus* Midichloriaceae**" and its main lineages, several of which are associated to aquatic organisms as well.

Conversely, the following organisms, ascribed by some authors to *Rickettsiales*, will not be treated here:

- Any organism in which, in the absence of a molecular characterization such as a partial 16S rRNA gene sequence, morphological and/or physiological similarities to members of *Rickettsiales* were observed (e.g., *Rickettsia*-like organisms (RLO) or *Ehlrichia*-like organisms) (e.g., Fryer and Lannan 1994; Michel et al.

1994). Indeed, we considered morphological features alone not sufficient, as clearly evidenced previously (e.g., Fryer et al. 1992; Tan and Owens 2000).
- The members of *Holosporaceae sensu lato* (Görtz and Schmidt 2005). Although several studies considered this group of obligate bacterial endosymbionts as the earliest divergent clade within *Rickettsiales*, more recently this phylogenetic positioning was put into doubt (Georgiades et al. 2011; Ferla et al. 2013; Schulz et al. 2014).
- The clade of free-living bacteria "*Candidatus* Pelagibacter," whose association to *Rickettsiales* was disproved (Brindefalk et al. 2011; Rodríguez-Ezpeleta and Embley 2012; Viklund et al. 2012).

The terminological notes for this chapter are:

- The term "symbiosis" and its derivatives are used according to the original definition by Anton De Bary (De Bary 1879), referring to organisms of different species living together, independently of the kind of interaction, therefore including indistinctly mutualism, commensalism, and parasitism. It should also be taken into consideration that in most cases presented below the role in the host physiology is currently unknown.
- All *Rickettsiales* are treated as "obligate endosymbionts" of eukaryotes; as currently, they are not cultivated in eukaryotic cell-free media. This does not imply that the association is necessary for the eukaryotic host.
- According to the rules of bacterial nomenclature, the formal description of a new species requires a sufficient set of characters, including metabolic features, and the availability of type material in public repositories. Since obligate bacterial endosymbionts are not cultivable, it is very difficult to meet such requirements, therefore the taxonomic status *Candidatus* has been created by Murray and Stackebrandt (Murray and Stackebrandt 1995). *Candidatus* definition requires only the phylogenetic position, as determined from some marker DNA sequence (typically the 16S rRNA gene), and the validation of authenticity of the bacterial identity from fluorescence in situ hybridization (or some equivalent technique). For these reasons, species (and higher taxonomic ranks) of obligate endosymbionts described thereafter generally fall into the *Candidatus* (abbreviated "*Ca*.") category, e.g., "*Ca*. Midichloria mitochondrii."

2 Aquatic *Rickettsiaceae*

According to the present knowledge, there are at least eight genus-level lineages of *Rickettsiaceae*, out of which six (highlighted in bold) present some representative associated to aquatic organisms (Fig. 3.1):

- ***Rickettsia***
- "***Ca*. Trichorickettsia**"

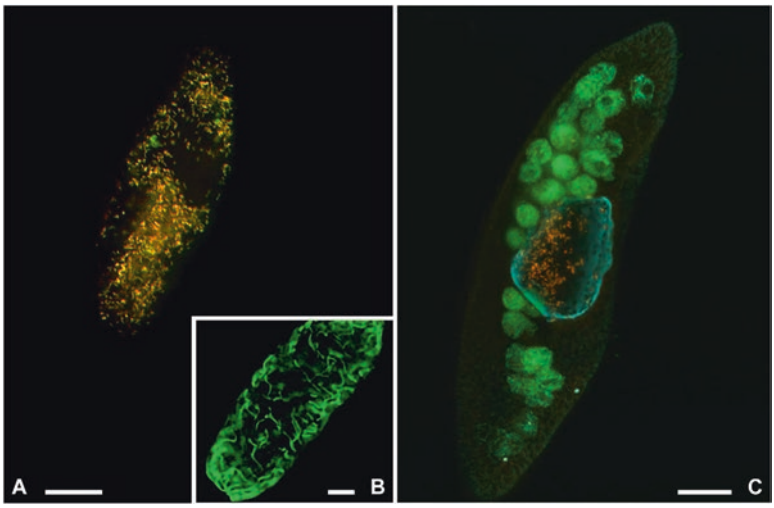

Fig. 3.3 Fluorescence in situ hybridization experiments performed on "*Candidatus* Trichorickettsia mobilis" and "*Candidatus* Gigarickettsia flagellata". "*Candidatus* Trichorickettsia mobilis" symbionts are located inside the cytoplasm of *P. nephridiatum* PAR (**a**) and inside the macronucleus of *P. multimicronucleatum* LSA (**c**), targeted by a specific probe (Cy3, *red signal*) together with a eubacterial probe (fluorescein, *green signal*). Food bacteria, labeled only in *green*, are visible inside food vacuoles in LSA. (**b**) Experiment performed on "*Candidatus* Gigarickettsia flagellata" endosymbiont of *S. minus* with a specific probe (*green signal*). Bars: 20 μm (Vannini et al. 2014, doi:10.1371/journal.pone.0087718.g003)

- "***Ca***. **Gigarickettsia**"
- "***Ca***. **Megaira**"
- *Orientia*
- "*Occidentia*"
- "***Ca***. **Cryptoprodotis**"
- "***Ca***. **Arcanobacter**"

Genus ***Rickettsia*** comprises two main clades (Fig. 3.1). The first clade includes well-known pathogens vectored by ticks, lice, and fleas, such as *Rickettsia prowazekii* and *Rickettsia typhi*, as well as endosymbionts of several other arthropods (Dumler et al. 2001; Perlman et al. 2006). Conversely, most members of the second clade of ***Rickettsia*** (mean identity with first clade ~96.4 %) were retrieved in association to aquatic organisms, i.e., three species of glossiphoniid leeches from Japan, namely *Torix tagoi*, *Torix tukubana,* and *Hemiclepsis marginata* (Kikuchi et al. 2002; Kikuchi and Fukatsu 2005), and the nucleariid amoeba *Nuclearia pattersoni* from Czech Republic (Dyková et al. 2003). In *T. tagoi*, the bacteria are present in the epidermis, esophagus, and intestine (Kikuchi et al. 2002). The *Rickettsia* endosymbionts of leeches are capable of vertical transmission, while their prevalence in natural host populations varies from 0 to 100 % (Kikuchi and Fukatsu 2005). The effect on leeches is unclear though some changes in animal size were observed (Kikuchi and Fukatsu 2005). It was speculated that, similarly to hematophagous

arthropods, the leech bite could transmit the rickettsial endosymbionts to vertebrates; however, preliminary PCR surveys on their major natural hosts (*Rana japonica*, *Rhinogobius brunneus*) were negative (Kikuchi and Fukatsu 2005), while the potential role of leeches as vectors for humans still needs to be clarified (Parola et al. 2015; Slesak et al. 2015).

"***Ca*. Trichorickettsia mobilis**" and "***Ca*. Gigarickettsia flagellata**" (Vannini et al. 2014) are phylogenetically closely related to the genus *Rickettsia* (Fig. 3.1); therefore, they can be especially useful to understand its evolutionary traits. Both endosymbiont species were retrieved exclusively in a number of ciliate protists, namely, *Paramecium multimicronucleatum*, *Paramecium nephridiatum,* and *Euplotes aediculatus* for "*Ca*. Trichorickettsia" and *Spirostomum minus* for "*Ca*. Gigarickettsia" (Fig. 3.3). The ciliate hosts were sampled from distantly related geographical areas such as Italy, Germany, and India.

"*Ca*. Gigarickettsia" is distinguishable for its unusually big size (rod-shaped, up to 20 μm long and 1.2 μm wide) and is located in the cytoplasm of the host *Spirostomum*. "*Ca*. Trichorickettsia" endosymbionts reside in the macronucleus in *P. multimicronucleatum*, whereas, in all other hosts, they have been retrieved in the cytoplasm. "*Ca*. Gigarickettsia" and "*Ca*. Trichorickettsia" hosted by *P. multimicronucleatum* display long (up to 10 μm) peritrichous flagella, which enable them of active movement inside the host cells, a unique case up to now among *Rickettsiales* (Fig. 3.2a). These *Rickettsiaceae* endosymbionts are also notable for two short (23–152 bp long) and closely located insertions in the 16S rRNA gene (*Escherichia coli* positions 207 and 212), with higher sequence divergence respect to the remaining gene sequence (90.7–100 % identity respect to 99.7–100 % in "*Ca*. Trichorickettsia") and showing some similarity to DNA polymerase III β subunit (Vannini et al. 2014).

Bacteria morphologically resembling "*Ca*. Trichorickettsia," including flagellar-driven motility, were previously found in the macronucleus of *P. multimicronucleatum* from the USA and Moldova although no molecular characterization is available (Vishnyakov and Rodionova 1999).

"***Ca*. Megaira**" genus forms a sister group to the clade of *Rickettsia*, "*Ca*. Trichorickettsia" and "*Ca*. Gigarickettsia" (Fig. 3.1). It is prominent among *Rickettsiaceae* for the abundance and distribution of its members and displays a great variety of eukaryotic host organisms, mostly unicellular, such as ciliates, green algae, and haplosporidians, but even multicellular, such as cnidarians (e.g., Hine et al. 2002; Vannini et al. 2005; Fraune and Bosch 2007; Sun et al. 2009; Sunagawa et al. 2009; Kawafune et al. 2012, 2014; Schrallhammer et al. 2013; Yang et al. 2016).

At least three "*Ca*. Megaira" subclades have been identified. The subclade of the type species "***Ca*. Megaira polyxenophila**" is the most abundant and richest in host species diversity (Schrallhammer et al. 2013). It has been found from multiple aquatic environments (freshwater, brackish, marine) worldwide (Italy, Germany, Denmark, the USA, Japan), in association with the free-living ciliates *Diophrys oligothrix*, *Euplotes octocarinatus*, *Paramecium caudatum,* and *S. minus* (Vannini et al. 2005; Schrallhammer et al. 2013), as well as with different kinds of green algae, such as the unicellular and colonial chlorophytes *Volvox carteri*, *Carteria*

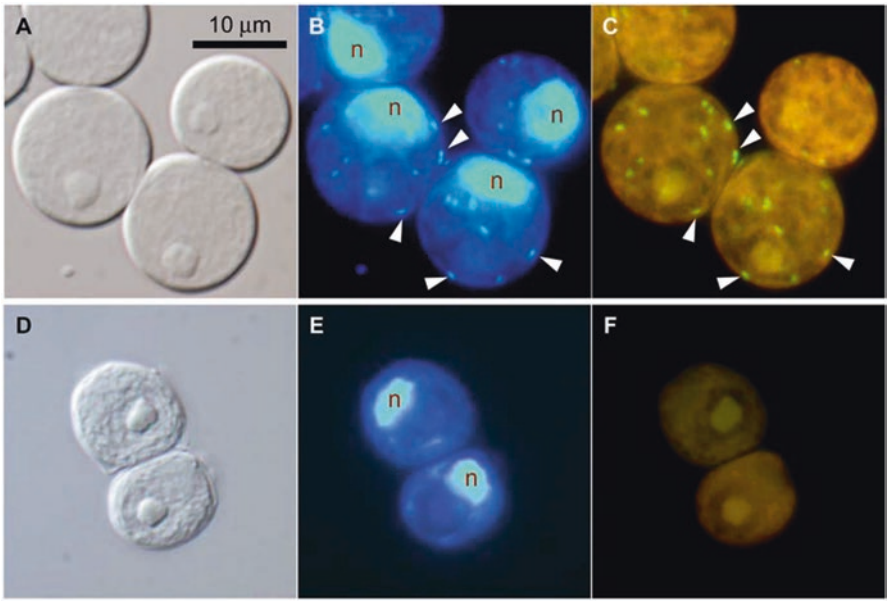

Fig. 3.4 Fluorescence in situ hybridization identification of endosymbionts in *C. cerasiformis* cells. (**a**–**c**) the endosymbiont-bearing *C. cerasiformis* strain NIES-425, (**d**–**f**) the endosymbiont-free *C. cerasiformis* strain NIES-424. Horizontal panels show the same cells, composed of Nomarski differential interference images (**a**, **d**), epifluorescence images with DAPI (4′,6-diamidino-2-phenylindole) staining (**b**, **e**), and epifluorescence images with a probe specific for the endosymbiont of *C. cerasiformis* NIES-425 (**c**, **f**). *Arrowheads* point to the signals from the endosymbionts. The *green signals* (**c**) represent endosymbiont-specific probes and the *yellow background* (**c**, **f**) is autofluorescence. All images are shown at the same magnification. The "n" indicates host cell nuclei (Kawafune et al. 2012, doi:10.1371/journal.pone.0031749.g003)

cerasiformis, and *Pleodorina japonica* (Fig. 3.4; Kochert and Olson 1970; Nozaki et al. 1989, 1994; Kawafune et al. 2012, 2014), and the unicellular streptophyte *Mesostigma viride* (Yang et al. 2016). "*Ca*. Megaira polyxenophila" is as rod-shaped bacterium of variable size, generally measuring 1.00–1.45 µm in length (elongated forms attain up to 3.2 µm) and 0.3 µm in width, with a typical Gram-negative structure (Kawafune et al. 2013; Schrallhammer et al. 2013; Yang et al. 2016). Similarly to *Rickettsia* (Silverman et al. 1978; Dumler and Walker 2005), "*Ca*. Megaira" cells are often observed surrounded by a clear electron-lucent (halo) layer and slime layer, without any host-derived membrane coating (Fig. 3.5). In most cases, the endosymbionts were harbored inside host cytoplasm, but in some ciliates (*P. caudatum* and *S. minus*) they resided in the host macronucleus (Schrallhammer et al. 2013).

The other two subclades of "*Ca*. Megaira" currently do not present any formally described species (Schrallhammer et al. 2013). Representatives of subclade B were found in association with the chlorophyte macroalga *Bryopsis* (Hollants et al. 2013), as well as to *Montastraea faveolata* (Sunagawa et al. 2009) and other corals (Penn et al. 2006). Members of subclade C were found as endosymbionts in the cytoplasm

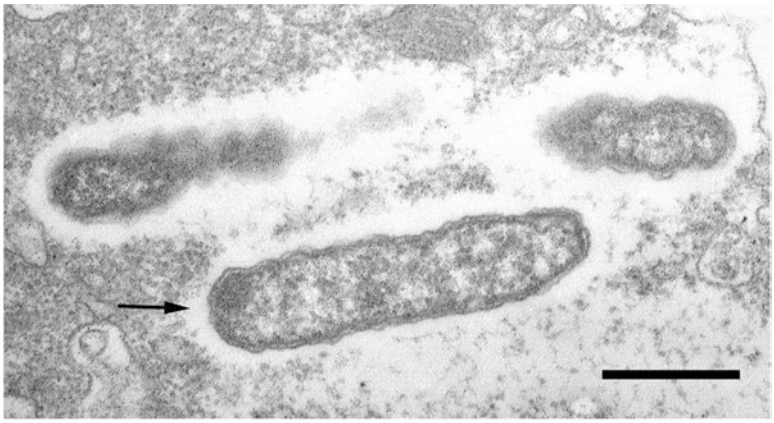

Fig. 3.5 Transmission electron microscopy of *D. oligothrix* BOD9 harboring "*Candidatus* Megaira polyxenophila." One whole bacterium and two partial ones are visible. Bacteria are free in the cytoplasm of the host. The *arrow* indicates the clear zone (halo) surrounding the cells. Bar: 1 μm (Schrallhammer et al. 2013, doi:10.1371/journal.pone.0072581.g004)

of ciliate *Ichthyophthirius multifiliis* (a fish parasite), where they measured up to 0.43 μm by 0.99 μm in size (Sun et al. 2009), and in the epithelial cells of the cnidarian *Hydra oligactis* (Fraune and Bosch 2007).

The tangled phylogenetic relationships among bacteria from different hosts within the genus "*Ca*. Megaira" are themselves a strong indication of several host transfers along their evolutionary history (Schrallhammer et al. 2013; Kawafune et al. 2015). The effect on their hosts is not evident, with only indirect information, for example, *C. cerasiformis* naturally infected strains grow slower than naturally noninfected (Kawafune et al. 2012), and "*Ca*. Megaira" was found in association with corals affected by white band disease (Sunagawa et al. 2009). Some recently produced molecular data may be helpful to investigate these aspects. In the study by Kawafune and colleagues (Kawafune et al. 2015), a horizontal gene transfer of DNA from "*Ca*. Megaira" into the nuclear genome of several strains of *V. carteri* which do not possess the endosymbiont was evidenced. The transferred DNA included, depending on the strain, partial sequences of "*Ca*. Megaira" 16S rRNA gene and of variable combinations of other genes involved in bacterial division and cell membrane and wall synthesis (Kawafune et al. 2015). On one side, these findings open to speculations on the functional significance of the phenomenon, with possible analogies with lateral transfer of *Wolbachia* DNA to the genomes of multiple hosts (Kondo et al. 2002; Dunning-Hotopp et al. 2007). Moreover, phylogeny of the horizontally transferred sequences was not fully consistent with the one of the algal host, further indicating a high level of host shift ability of "*Ca*. Megaira," even respect to closely related host organisms (Kawafune et al. 2015).

In the transcriptomic study of the streptophyte *M. viride* hosting "*Ca*. Megaira polyxenophila," 91 genes were putatively assigned to the endosymbiont (Yang et al. 2016). Some of them were homolog to *Rickettsia* genes involved in host invasion

and interaction, such as the surface proteins Sca4, Sca5/rOmpB, a component of VirB type IV secretion system, and ankyrin domain proteins (Gillespie et al. 2010; Renvoisé et al. 2011; Sears et al. 2012). In *Rickettsia*, the adhesion between the bacterium and host through surface proteins involves rOmbB and induces actin-based cytoskeletal rearrangements in the host, resulting in the entry of the bacterial cell (Martinez and Cossart 2004; Chan et al. 2010). Although *M. viride* is not known to possess active phagocytotic activity, in its transcriptome were retrieved the homologs for Ku70, receptor for rOmpB of *Rickettsia*, and for genes involved in actin-based cytoskeletal rearrangements (Yang et al. 2016). It was therefore possible to hypothesize that "*Ca*. Megaira" might be able to induce phagocytosis in order to enter the host alga via active engulfment, in a analogous way to what *Rickettsia* does, for example, in endothelial cells (Chan et al. 2010).

Another relevant feature of "Ca. Megaira" is the frequent coexistence with other bacterial symbionts in the same host, such as the betaproteobacterium *Polynucleobacter necessarius* in *E. octocarinatus*, a member of the order *Sphingobacteriales* in *Ichthyophthirius multifiliis*, and the member of "*Ca*. Midichloriaceae" "*Ca*. Bandiella" in *H. oligactis* (Fraune and Bosch 2007; Sun et al. 2009; Schrallhammer et al. 2013).

"***Ca*. Cryptoprodotis polytropus**" was found in an endosymbiotic relationship with the ciliate *Pseudomicrothorax dubius* (Ferrantini et al. 2009). It occupies an early divergent position within the family (88.8–90.5 % similarities with most other members), possibly more closely related to *Orientia tsutsugamushi* and other endosymbionts of arthropods though with limited support (Fig. 3.1). The endosymbiont cells were shown to be rod shaped, attaining up to 3 μm in length and 0.5 μm in width, and abundant in the cytoplasm of the host.

"***Ca*. Arcanobacter lacustris**" is the earliest diverging known member of the family (Fig. 3.1; identity in the approximate range 88.9–91.6 %) sampled from Damariscotta Lake water (Maine, USA) (Martijn et al. 2015). The draft assembly of the partial genome sequence of this bacterium, whose putative host was not identified, was obtained through multiple displacement amplification, and amounts to 822,563 bp (151 contigs; 32.6 % GC, 88.4 % coding density), with a predicted genome size of 1.7 Mb, which is larger than most other *Rickettsiaceae* (http://www.ncbi.nlm.nih.gov/genome). Some genes encoded are typical in *Rickettsiaceae* and other *Rickettsiales*, such as an ATP/ADP translocase, which can be employed to uptake energetic nucleotides from the host (Schmitz-Esser et al. 2004; Audia and Winkler 2006), and components for a VirB type IV secretion system translocator (Gillespie et al. 2010), together with a set of 18 putative effectors possibly involved in the interaction with the host, such as ankyrin repeat proteins, phosphoglucomutases, and Fic-family proteins (Gillespie et al. 2015). The gene repertoire of "*Ca*. Arcanobacter" appears richer and peculiar respect to its relatives (299 out of 723 ortholog clusters unique within *Rickettsiales*). The most relevant genomic features compared to other representatives of *Rickettsiaceae* are 22 genes for flagellar assembly (components for hook, filament, basal body, and motor) and 9 genes for bacterial chemotaxis. Other unique genes within the family are those involved in threonine metabolism, glycolysis, pantothenate and CoA biosynthesis, terpenoid backbone synthesis, and transporters for thiamine and antibiotics (Martijn et al. 2015).

3 Aquatic *Anaplasmataceae*

The family *Anaplasmataceae* encompasses at least seven genus-level lineages (plus the genus *Aegyptianella*, associated to terrestrial organisms and considered by Dumler and coauthors as *Anaplasmataceae incertae sedis* (Dumler et al. 2001), which is not taken into account since the 16S rRNA gene sequence available is only ~600 bp; Rikihisa et al. 2003). Three of these genera (highlighted in bold) were retrieved in association with aquatic organisms, all multicellular (metazoans), making *Anaplasmataceae* the only *Rickettsiales* family without known protist hosts so far (Fig. 3.1):

- *Anaplasma*
- *Ehrlichia*
- "*Ca*. Neoehrlichia"
- **"*Ca*. Xenohaliotis"**
- *Wolbachia*
- ***Neorickettsia***
- **" Ca. Xenolissoclinum"**

Members of the genus ***Neorickettsia***, such as ***Neorickettsia sennetsu*** and ***Neorickettsia helminthoeca***, are known to be associated to aquatic organisms such as salmons and trematodes, which can be vectors for the disease. Genus *Neorickettsia* is treated in a separate chapter of this book (Chap. 15).

"***Ca***. **Xanohaliotis californiensis**" is an endosymbiont of abalone gastropods *Haliotis* spp. and is considered the etiological agent of the withering syndrome (Friedman et al. 2000; Crosson et al. 2014). This disease is characterized by atrophy and metaplasia of digestive gland, depletion of glycogen reserves, atrophy of pedal muscle due to its digestion as energy source, and eventual death (Kismohandaka et al. 1993; Gardner et al. 1995). The endosymbiont resides within basophilic cytoplasmic vacuoles of abalone gastrointestinal epithelial cells, usually situated apical to their nucleus. It is nonmotile, and predominantly displays a rod shape (0.33 × 1.55 μm on average) with a pleomorphy to cocco-bacillar or even spherical shape (Friedman et al. 2000). Peptidoglycan and slime layers were not detected, in compliance with features of other *Anaplasmataceae* (Dumler and Walker 2005).

In addition, it was found that the presence of a phage hyperparasite, morphologically resembling Siphoviridae (Caudovirales) (Cruz-Flores and Cáceres-Martínez 2016), can reduce pathogenicity of "*Ca*. Xenohaliotis" and lethality of the disease (Friedman and Crosson 2012).

Due to ecological and economical importance of abalone, the withering syndrome has relevant impact and was the subject of several studies (Crosson et al. 2014). "*Ca*. Xenohaliotis" was originally retrieved along the eastern Pacific margin of North America, although, due to transport of infected abalone, the present geographical range appears much larger (Friedman et al. 2000; Crosson et al. 2014). Since temperature is a major factor influencing transmission and development of the disease (Moore et al. 2000; Braid et al. 2005), climatic changes or even short-term ocean temperature variations can produce significant effects on health conditions of natural abalone populations (Neuman et al. 2010).

Several tools are available to identify and quantify "*Ca*. Xenohaliotis," such as specific in situ hybridization and real-time PCR assays (Antonio et al. 2000; Friedman et al. 2014).

"***Ca*. Xenolissoclinum pacificiensis**" was retrieved in association with the ascidian tunicate *Lissoclinum patella* collected near the coast of Papua New Guinea (Kwan and Schmidt 2013). Analysis of 16S rRNA gene amplicon abundance in several *L. patella* animals led the authors to hypothesize a natural tripartite interaction, involving also "*Ca*. Endolissoclinum faulkneri," another *Thalassobaculum*-related alphaproteobacterial endosymbiont commonly residing in the ascidian zooids. In the case of first *L. patella* animal analyzed, the complex and not yet uncovered dynamics of the interaction would have resulted in a complete displacement of "*Ca*. Endolissoclinum" by "*Ca*. Xenolissoclinum."

A draft genome of "*Ca*. Xenolissoclinum" was produced (nine contigs; 1.04 Mb, 32.1 % GC) (Kwan and Schmidt 2013). Some features were found to be consistent with other *Anaplasmataceae*, such as the lack of a functional LPS (lipopolysaccharide) biosynthetic pathway, and with *Rickettsiales* in general, such as a VirB type IV secretion system apparatus, as well as a predicted effector protein containing ankyrin repeats. As distinctive feature in respect to the other representatives of the family, genes involved in flagellar assembly such as hook, ring and rod were present.

4 "*Ca*. Midichloriaceae"

The discovery of the family "*Ca*. Midichloriaceae" is probably among the most significant advances in the knowledge of *Rickettsiales* diversity produced in the last 20 years. Its first representatives that were molecularly characterized were retrieved in association with amoebas and ticks (Fritsche et al. 1999; Parola et al. 2003; Mediannikov et al. 2004). Already in these earliest reports, it was hypothesized that the new *Rickettsiales* lineage obtained in phylogenetic analysis would deserve a family status. This point became clearer in the following years, after the identifications of several other organisms belonging to this lineage, in particular the tick endosymbiont "*Ca*. Midichloria mitochondrii" (Sacchi et al. 2004; Sassera et al. 2006; Epis et al. 2008). Recently, a formal taxonomic description of the "*Ca*. Midichloriaceae" family was produced (Montagna et al. 2013). The family forms a well supported and independent lineage clustering together with *Rickettsiaceae* and *Anaplasmataceae* (Fig. 3.1). Although the evolutionary relations among the three families are sometimes conflicting in literature, more recent data tend to support the topology shown in Fig. 3.1, in which *Anaplasmataceae* and "*Ca*. Midichloriaceae" are sister groups (e.g., Driscoll et al. 2013; Wang and Wu 2014a; Schulz et al. 2016). It was also shown that, in terms of 16S rRNA gene sequence variability and of host taxonomic range, the diversity of this new family is at least comparable with *Rickettsiaceae* and *Anaplasmataceae* (Montagna et al. 2013). Indeed "*Ca*. Midichloriaceae" were retrieved in association with a variety of eukaryotic hosts,

spanning from ticks and other terrestrial arthropods to several aquatic organisms, such as ciliates, amoebas, cnidarians, sponges, euglenids, placozoans, echinoderms, fish, and possibly even to humans and other mammals (e.g., Fritsche et al. 1999; Mediannikov et al. 2004; Sassera et al. 2006; Fraune and Bosch 2007; Longford et al. 2007; Epis et al. 2008; Lloyd et al. 2008; Sipkema et al. 2009; Sunagawa et al. 2010; Vannini et al. 2010; Mariconti et al. 2012a; Matsuura et al. 2012a; Bazzocchi et al. 2013; Boscaro et al. 2013a, b; Driscoll et al. 2013; Kimes et al. 2013; Kuo and Lin 2013; Szokoli et al. 2016).

The evolutionary relationships among different representatives of "*Ca*. Midichloriaceae" do not appear well resolved for the moment, since phylogenetic analyses performed on 16S rRNA gene sequences, the only marker available in most cases, in general fail to recover consistent and well supported clades. This was interpreted as due to high and uneven rates of sequence evolution in this clade (e.g., Vannini et al. 2010; Boscaro et al. 2013a, b; Szokoli et al. 2016). It is anyway possible to identify some well-supported lineages, especially those for which multiple endosymbiont sequences are available ("*Ca*. Midichloria," "*Ca*. Lariskella," "*Ca*. Bandiella") (Epis et al. 2008; Matsuura et al. 2012a; Senra et al. 2016).

Currently, the capabilities of representatives of "*Ca*. Midichloriaceae" to produce pathogenic effects in humans or other vertebrates has not been fully demonstrated; nevertheless some studies provide quite strong indications in that sense (See Chap. 14).

Only three genomes are sequenced for the members of the family "*Ca. Midichloriaceae*"; viz., "*Ca*. Midichloria mitochondrii" (Sassera et al. 2011) and two different strains of "*Ca*. Jidaibacter acanthamoeba" (Wang and Wu 2014b; Schulz et al. 2016), as well as fragmented partial sequences from the genome of "*Ca*. Bandiella sp." (Driscoll et al. 2013).

Currently, nine genera of "*Ca*. Midichloriaceae" are recognized, all of which are presented in detail below (in bold those associated with aquatic organisms):

- "*Ca*. Midichloria"
- "*Ca*. Lariskella"
- "***Ca*. Bandiella**"
- "***Ca*. Jidaibacter**"
- "***Ca*. Anadelfobacter**"
- "***Ca*. Cyrtobacter**"
- "***Ca*. Defluviella**"
- ***Lyticum***
- "***Ca*. Fokinia**"

"***Ca*. Midichloria mitochondrii**" was the first representative of the family which received a formal description (Sassera et al. 2006) and is probably the most studied. It was originally observed in the ovaries (oocytes, luminal and funicular cells) of the sheep tick *Ixodes ricinus* (Lewis 1979; Zhu et al. 1992; Sacchi et al. 2004). In the cytoplasm, it measures approximately 0.45 × 1.2 μm and can be surrounded by a host-derived membrane (Beninati et al. 2004). It displays the distinctive ability to invade host mitochondria, locating between the two membranes, and to multiply

there consuming the matrix, although without apparent harm for the tick. This is a unique feature among known endosymbionts of multicellular organisms (Sacchi et al. 2004). More recently, "*Ca*. Midichloria mitochondrii" was retrieved in lower amounts in salivary glands of *I. ricinus*, and, interestingly, DNA and proteins from this endosymbiont were evidenced also in blood of humans and other mammals bitten by ticks (Skarphédinsson et al. 2005; Mariconti et al. 2012a; Bazzocchi et al. 2013) (see Chap. 14).

Consistently with the localization in the oocytes, "*Ca*. Midichloria mitochondrii" is vertically transmitted with 100 % efficiency. In the progeny, the bacteria are localized predominantly in primordial sex organs of both sexes and are retained in females, while in males they decrease in number and can be lost completely (Lo et al. 2006; Sassera et al. 2008; Epis et al. 2013). Indeed, the prevalence of "*Ca*. Midichloria mitochondrii" in natural populations of *I. ricinus* was found at 100 % in females and at 44 % in males by PCR analyses although it can be lost even by some females after few generations in laboratory conditions (Lo et al. 2006).

The relationship with the tick host was not fully clarified, the most probable hypothesis on the available data indicates a facultative mutualism (Lo et al. 2006). The quantitative variations in prevalence during host life cycle (i.e., increased load after engorgement and decreased after molting) suggest that the role of "*Ca*. Midichloria" is dependent of host life stages (Sassera et al. 2008). A tetracycline treatment reduced the extent of such increase in endosymbiont load although it did not eliminate the bacterium completely (Ninio et al. 2015).

Sequences of 16S rRNA gene affiliated to "*Ca*. Midichloria" in several continents (such as Europe, Asia, Northern and Southern America) were determined. The host organisms were in most cases hard ticks, such as *I. ricinus*, other *Ixodes* species, *Amblyomma* spp., *Dermacentor* spp., *Haemaphysalis* spp., *Hyalomma* spp., and *Rhipicephalus* spp. (Parola et al. 2003; Lo et al. 2006; Loftis et al. 2006; Špitalská et al. 2008; Epis et al. 2008; Hornok et al. 2008; Van Overbeek et al. 2008; Venzal et al. 2008; Seng et al. 2009; Dergousoff and Chilton 2011; Harrus et al. 2011; Tijsse-Klasen et al. 2011; Subramanian et al. 2012; Williams-Newkirk et al. 2012, 2014; Smith et al. 2013; Tveten et al. 2013; Granquist et al. 2014; Palomar et al. 2015; Klubal et al. 2016), but even mites (*Spelaeorhynchus praecursor*) (Reeves et al. 2006) and insects such as *Cimex lectularius* (Richard et al. 2009) and *Tabanus tergestinus* (Hornok et al. 2008). The host and endosymbiont phylogenies are not fully congruent, even within the same host species, strongly suggesting the ability of horizontal transmission of "*Ca*. Midichloria", possibly mediated by the blood of vertebrates bitten by the ticks (Epis et al. 2008; Williams-Newkirk et al. 2012).

The localization of "*Ca*. Midichloria" was investigated in only two tick species other than *I. ricinus*. In *Rhipicephalus bursa*, it was found in the ovaries, in the cytoplasm without surrounding vacuoles, but also in the mitochondria, although without signs of matrix degeneration, with a cell size of 1 × 0.25 μm (Epis et al. 2008). In *Ixodes holocyclus*, it was observed as rod-shaped bacteria measuring around 0.5 × 1.2–1.8 μm in the cytoplasm of ovary cells, but not in mitochondria, which was hypothetically related to the small size of the organelle in this tick spe-

cies (Beninati et al. 2009). It should also be taken into account that the even corresponding 16S rRNA gene sequence is relatively divergent (around 97–98 % identity, which is even below the commonly accepted species threshold of 98.65–98.7 % (Stackebrandt and Ebers 2006; Kim et al. 2014) respect to "*Ca*. Midichloria mitochondrii" from *I. ricinus*).

The genome of "*Ca*. Midichloria mitochondrii" endosymbiont of *I. ricinus* was the first among "*Ca*. Midichloriaceae" family to be sequenced (1,183,732 bp circular, GC content: 36.6 %; Sassera et al. 2011). The main findings were the retrieval for the first time within *Rickettsiales* of 26 genes putatively encoding all key components (hook, filament, basal body) of a flagellum, which were also shown to possess the conserved typical domain structure (Mariconti et al. 2012b), and of a cbb3 cytochrome oxidase complex. Both gene sets were consistent with vertical transmission from a *Rickettsiales* common ancestor according to phylogeny and GC content (Sassera et al. 2011). Six flagellar genes and three cbb3 genes were found to be expressed at RNA level during different life stages of the tick, in particular the six flagellar ones were co-expressed in the eggs and adult ovaries (Sassera et al. 2011; Mariconti et al. 2012b). Additionally, the flagellar protein FliD of "*Ca*. Midichloria mitochondrii," i.e., the external flagellar cap, was detectable by western blot and was shown to be localized on the surface of bacterial cells (Mariconti et al. 2012b). It was speculated that flagellar components may also be involved in other functions related to host invasion as secretory apparatus, considering the homologies shared with components of type III secretion systems (Mariconti et al. 2012b). On the other side, the cbb3 oxidase can reduce oxygen with lower efficiency but higher substrate affinity compared to the caa3 oxidase, which is typical in most other *Rickettsiales* but, interestingly, present only as a residual gene fragment in "*Ca*. Midichloria mitochondrii." It was hypothesized by the authors that cbb3 oxidase could be involved in a beneficial effect for the host, enabling the endosymbiont to produce ATP at oxygen concentrations that would be suboptimal for the mitochondrion. The ATP could then be provided directly to the tick via the ATP/ADP translocase of "*Ca*. Midichloria mitochondrii" (Sassera et al. 2011).

Similarly to *Rickettsiales*, other metabolic pathways appear to be reduced in "*Ca*. Midichloria mitochondrii," which nevertheless maintained the capability to synthesize several cofactors, including coenzyme A, biotin, lipoic acid, tetrahydrofolate, pantothenate, heme, and ubiquinone, as well as functional pathways for gluconeogenesis, partial glycolysis, and Krebs cycle (Sassera et al. 2011). Also genes that are likely involved in interaction with the host, such as those encoding for VirB type IV and Sec-independent protein secretion systems, as well as for ankyrin repeat putative effector proteins, were found in the genome (Sassera et al. 2011).

Another prominent group of "*Ca*. Midichloriaceae" is represented by "***Ca*. Lariskella arthropodarum**" (Matsuura et al. 2012a, b). This endosymbiont was reported in association with several arthropods, including a number of hemipterans such as *Nysius* spp., *Kleidocerys resedae*, *Arocatus melanostomus*, *Dimorphopterus pallipes*, *Horridipamera inconspicua*, *Paromius exiguus*, *Physopelta* spp., *Neuroctenus castaneus*, *Dolycoris baccarum*, *Eysarcoris* spp., and *Piezodorus hybneri* (Matsuura et al. 2012a; Takeshita et al. 2015), fleas such as *Xenopsylla cheopis*

and *Ctenocephalides felis* (Erickson et al. 2009), coleopterans such as *Curculio morimotoi* and *Curculio okumai* (Toju et al. 2013), and ticks such as *Ixodes* spp. and *Haemaphysalis concinnae* (Mediannikov et al. 2004; Eremeeva et al. 2006, 2007; Fujita et al. 2007; Qiu et al. 2014; Kurilshikov et al. 2015). According to the reports available, the distribution of "*Ca*. Lariskella" was retrieved in Asia and Eastern Europe(Western Russia, Estonia). The prevalence of this endosymbiont was investigated in some natural populations of different hosts. In hemipterans, it was observed in the range 77.6-100 %, with variations also among populations of the same host species (Matsuura et al. 2012a). In the tick *Ixodes persulcatus*, somehow similarly to "*Ca*. Midichloria mitochondrii" in *I. ricinus*, it was found to be more abundant in females (up to 71.43 %), where it can constitute ~40 % of the total microbiome, than in males (up to 16 %) (Eremeeva et al. 2006, 2007; Kurilshikov et al. 2015).

"*Ca*. Lariskella" cells were observed as small filamentous bacteria in bacteriomes, ovaries (nurse cells, follicular cells, and oocytes), and midgut of *Nysius plebeius* and *K. resedae*, in coexistence with *Wolbachia* and, respectively, the gammaproteobacterial endosymbionts "*Ca*. Scheideria nysicola" and "*Ca*. Kleidoceria schneideri." The observation in oocytes suggests vertical inheritance of this endosymbiont (Matsuura et al. 2012a).

Consistently with the recent retrieval of "*Ca*. Lariskella" from tick salivary glands (Qiu et al. 2014), there is evidence of transmission to human subjects of DNA from this endosymbiont after a tick bite, associated with some pathogenic effects (Mediannikov et al. 2004) (see Chap. 14).

The "***Ca*. Bandiella**" clade is strongly supported in phylogeny (Fig. 3.1) and represents a peculiar case among "*Ca*. Midichloriaceae" for its broad host range, both in taxonomic and geographical sense, somehow reminiscently of "*Ca*. Megaira" (Senra et al. 2016). Indeed, members of this clade were retrieved in association with several marine and freshwater organisms, both unicellular such as ciliates (*Euplotes woodruffi* and *Condylostoma spatiosum*) (Senra et al. 2016; Gong et al. 2016) and euglenids (*Eutreptiella* sp.) (Kuo and Lin 2013), as well as metazoans such as sponges (*Cymbastela concentrica*) (Longford et al. 2007), placozoans (*Trichoplax adhaerens*) (Driscoll et al. 2013), echinoderms (*Aposticopus japonicus*) (JX170254 Gao et al., unpublished), and cnidarians (*H. oligactis* and corals, including *M. faveolata* and *Gorgonia ventalina*) (Fraune and Bosch 2007; Sunagawa et al. 2009, 2010; Kimes et al. 2013; Frade et al. 2016). The relatively high sequence diversity (up to 90.4 %), which is even below the formal genus threshold of 94.5 % proposed by Yarza and coauthors (Yarza et al. 2014), suggests that several distinct species could belong to the "*Ca*. Bandiella" clade (Senra et al. 2016). Up to now, the only formally described was **"*Ca*. Bandiella woodruffii"**, a rod-shaped endosymbiont of the ciliate *E. woodruffi* from Brazil, in coexistence with the betaproteobacterium *P. necessarius* (Senra et al. 2016). Some hints for an unstable horizontal symbiont transfer towards another *Euplotes* strain were presented in the same study.

Other detailed accounts were produced for the endosymbionts of *Eutreptiella* sp., *H. oligactis*, and *T. adhaerens*. In both *Eutreptiella* and *H. oligactis*, bacteria were observed enclosed in host-derived vacuoles, individually or in groups, each

cell measuring 2–4.5 μm (Kuo and Lin 2013; Fraune and Bosch 2007). The endosymbiont did not appear harmful for *Eutreptiella*, which was also unable to consume it as energy source (Kuo and Lin 2013). As previously mentioned, in *H. oligactis* "*Ca*. Bandiella" was observed in the epithelial cells in coexistence with "*Ca*. Megaira," and its association with the host was stable for several decades in laboratory conditions (Fraune and Bosch 2007). Some partial genomic data (roughly estimated at 20 %) were made available for the "*Ca*. Bandiella" associated with *T. adhaerens* by the extraction from a host genome assembly of protein-coding sequences putatively assigned to the endosymbiont, termed by the authors as RETA (*Rickettsiales* endosymbiont of *T. adhaerens*) (Driscoll et al. 2013). Interestingly, few components of the bacterial flagellar system were found, which are consistent with "*Ca*. Midichloriaceae" phylogeny. The presence of parA homolog was interpreted as a possible indication for the presence of a plasmid, similarly to some *Rickettsia* (Baldridge et al. 2010). Other relevant features encoded in the partial gene set of the RETA genome include components of VirB type IV secretion system, other proteins putatively involved in endosymbiosis-related functions, such as host invasion, intracellular growth and survival, metabolite uptake from the host, as well as DNA integration/conjugation. This "*Ca*. Bandiella" endosymbiont likely corresponds to a vertically transmitted Gram-negative bacterium previously observed within the fiber cells of *T. adhaerens* (Eitel et al. 2011).

"***Ca*. Jidaibacter acanthamoeba**" was retrieved as bacterial endosymbiont with variable shape (straight to curved, 0.3–0.5 × 0.8–2.3 μm) in two strains of *Acanthamoeba* sp. (UWC8 and UWC36), isolated from the cornea of human patients with keratitis (Fritsche et al. 1999). It represented the first molecular characterization of a symbiont associated to protists in the entire order *Rickettsiales* (excluding *Holosporaceae*). Both strains of endosymbionts could be transferred to related amoeba strains in laboratory experiments and may have a moderate negative influence on host growth (Gautom and Fritsche 1995; Schulz et al. 2016).

Genome sequencing was independently performed for both endosymbiont strains (Wang and Wu 2014b; Schulz et al. 2016) and also led to the formal *Candidatus* species description (Schulz et al. 2016). Despite a high 16S rRNA gene sequence identity (~99.6 %), the two genomes differ remarkably in size (UWC8: 1.6 Mb circular, 34.7 % GC; UWC36: 144 contigs, 2.4 Mb, 34 % GC), nevertheless no direct detailed comparison of their contents is available. The large size of the UWC36 genome and the high number of genes shared with free-living *Alphaproteobacteria* rather than *Rickettsiales* suggest that this endosymbiont retained several ancestral features and even led the authors to hypothesize that it may represent a missing link between free-living and symbiotic *Alphaproteobacteria* (Schulz et al. 2016), with some analogies with other groups of intracellular bacteria hosted by amoebae such as *Chlamydiae* (Horn 2008). More than 10 % of the UWC36 encoded proteins are putative effectors containing eukaryotic-like repeat domains, such as, among several others, ankyrin, leucine-rich, and tetratricopeptide repeats. These may exert a hijacking role in several host functions, for example, inhibiting phagosome–lysosome fusion, similarly to *Rickettsiales* and other intracellular bacteria such as *Legionella* (Al-Quadan et al. 2012; Gillespie et al. 2015). In line with this observation, three

different type IV secretion system clusters, namely VirB, Tra, Trb, the latter unusual within the order, were retrieved. Another relevant feature of UWC36 genome is the highest number (35) of flagellar genes among *Rickettsiales*, despite flagella not being observed. Interestingly, phylogenetic analyses are consistent with vertical inheritance for flagellar genes and VirB system, while for Tra and Trb they would suggest horizontal gene transfers, possibly from distantly related bacteria (Schulz et al. 2016).

Other predicted metabolic features, which are comparable to other *Rickettsiales*, were found in both "*Ca*. Jidaibacter" strains (Wang and Wu 2014b; Schulz et al. 2016). These shared pathways include Krebs cycle, electron transport chain, pentose phosphate pathway, and gluconeogenesis, while key glycolysis enzymes are absent. Some biosynthetic deficiencies were evidenced and are likely compensated by a rich set of membrane transporters, including a putative ATP/ADP translocase. Moreover, consistently with ultrastructural observations of a Gram-negative structure of the endosymbionts cells, which were surrounded by a clear adjacent zone suggestive of capsules or slime layers (Fritsche et al. 1999), complete pathways were found for synthesis of peptidoglycan, LPS, and even, a unique case among *Rickettsiales*, of the capsule (Wang and Wu 2014b; Schulz et al. 2016).

The genus ***Lyticum***, a long time known endosymbiont of *Paramecium* spp. (Preer et al. 1974; Preer and Preer 1982), was recently affiliated to the "*Ca*. Midichloriaceae" thanks to molecular characterization (Boscaro et al. 2013a). The two formerly recognized species were maintained although their 16S rRNA gene similarity (99.5 %) was shown to be higher than the threshold of 98.65–98.7 % (Stackebrandt and Ebers 2006; Kim et al. 2014). The type species ***Lyticum flagellatum*** occurs in the cytoplasm of *Paramecium octaurelia* and *Paramecium tetraurelia* from the USA and Panama and displays a straight rod shape (0.6–0.9 × 2.0–4.0 μm) (Jurand and Preer 1969). Conversely, ***Lyticum sinuosum*** was retrieved in the cytoplasm of *Paramecium biaurelia* from the USA and has a curved shape, up to 1.1 × 7.8 μm (Preer et al. 1974). Both *Lyticum* species are covered by numerous peritrichous flagella about 4 μm long (Jurand and Preer 1969; Preer et al. 1974; Boscaro et al. 2013a), representing the only case in which a distinct flagellar structure was observed within "*Ca*. Midichloriaceae" (Fig. 3.2b). Nevertheless, no flagellar-mediated movement was observed, so the flagella were even hypothesized to possibly have some alternative role in establishment and maintenance of the endosymbiosis (Boscaro et al. 2013a). *Lyticum* species show a Gram-negative ultrastructure, and are enclosed in host membrane-bound vesicles, where the bacteria are often found to reside in small groups (Jurand and Preer 1969; Preer et al. 1974). Some earlier investigations reported a killer effect exerted by *Paramecium* strains harboring *Lyticum* spp. towards endosymbiont-free paramecia (Sonneborn 1959; Jurand et al. 1982), but these findings were not confirmed in more recent studies (Boscaro et al. 2013a), and appear still somehow controversial. *L. flagellatum* was also hypothesized to provide folate to its ciliate host (Soldo and Godoy 1973).

The other described members of the family are all endosymbiont of ciliate protists, for which limited data, in the form of 16S rRNA gene sequence and intracellular localization in the host by FISH or TEM, is available.

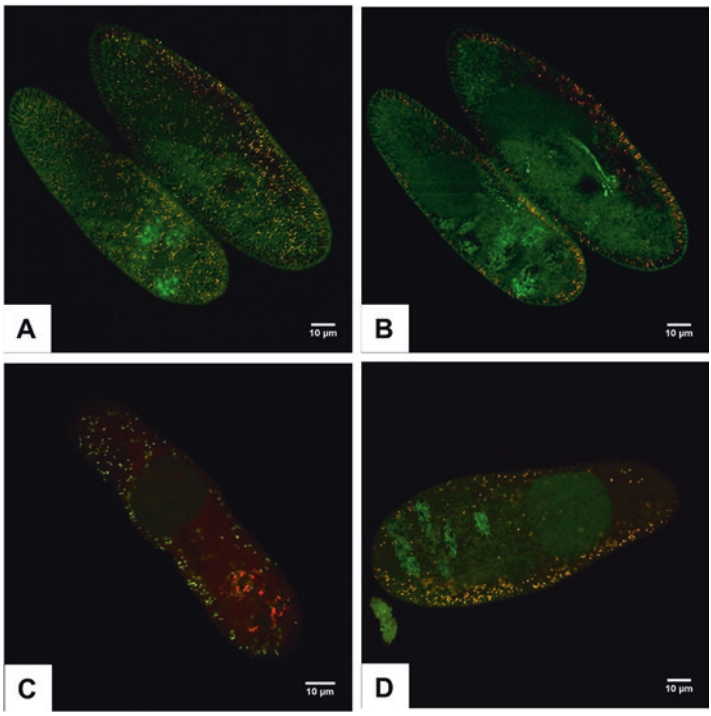

Fig. 3.6 Species-specific in situ detection of "*Candidatus* Fokinia solitaria" in *Paramecium* sp. strain Rio ETE ALG 3VII. (**a**, **b**, **d**) Merge of the signals from eubacterial probe (fluorescein-labelled, *green signal*) and two different (**a**, **d**) species-specific probes or (**b**) an alphaproteobacterial probe (Cy3-labelled, *red signal*), thus "*Candidatus* Fokinia solitaria" appears *yellowish* due to merging of the two signals. In (**c**), the signal of a third species-specific probe is shown in *green*. Stratification of the endosymbiont in section through the host cortex (**a**, **c**) and through the inner part of the host cell (**b**, **d**). Bars: 10 µm (Szokoli et al. 2016, doi:10.1371/journal.pone.0145743)

"***Ca***. **Anadelfobacter veles**" was found as cytoplasmic endosymbiont of a *Euplotes harpa* strain in Italy (Vannini et al. 2010). This bacterium is rod-shaped, from 5 to 6 µm long and from 1 to 2 µm wide. Its cytoplasm is electron dense, and the cell was found to be often surrounded by a clear halo, and in some cases a host-derived symbiosomal membrane was visible.

The genus "***Ca***. **Cyrtobacter**" includes two species. "***Ca***. **Cyrtobacter comes**" was found as endosymbiont of *E. harpa* from Denmark (Vannini et al. 2010). Its cells frequently showed an irregular shape, measuring about 4–5 µm in length and 2–3 µm in width. They were not surrounded by any host membrane although an adjacent clear zone was often visible. "***Ca***. **Cyrtobacter zanobii**" was identified as rod-shaped bacterial endosymbiont of a strain of *E. aediculatus* from India (Boscaro et al. 2013b).

"***Ca***. **Defluviella procrastinata**" is a short rod-shaped endosymbiont of *P. nephridiatum* isolated from a wastewater treatment plant in Italy (Boscaro et al. 2013b).

"***Ca***. **Fokinia solitaria**" is a rod-shaped (1.2 μm long and 0.25–0.35 μm wide) Gram-negative endosymbiont of *Paramecium* sp. isolated from a wastewater treatment plant in Brazil (Szokoli et al. 2016). Its cells are prevalently stratified in a narrow layer in the cytoplasmic cortex between the trichocysts or just below them, mostly parallel to their axis (Fig. 3.6). They are not enveloped by host membrane. No flagella were observed although some features (in particular, a narrow fibrillar layer lacking host ribosomes around bacteria and a tail of trailing material) would have been compatible with their presence. The endosymbionts were also quite frequently observed enclosed in host autolysosomes. This finding was interpreted by the authors as a possible host defense mechanism towards "*Ca*. Fokinia," and the prevalent cortical location of the bacterial cells would represent a way to be protected from such host response.

"*Ca*. Defluviella" and "*Ca*. Fokinia" are closely related in phylogenetic analysis (Fig. 3.1) and interestingly share four short nucleotide insertions in their 16S rRNA gene in homolog positions, which are predicted to increase the length of two stems in the 16S rRNA (Szokoli et al. 2016).

Several other sequences 16S rRNA gene sequences affiliated to "*Ca*. Midichloriaceae" were obtained in different types of screening studies (e.g., Walsh et al. 2009; Zhang et al. 2013, 2014), in most cases from aquatic environments. Among them, there are some notable cases. For example, an organism phylogenetically close to "*Ca*. Midichloria mitochondrii" (~96 % identity, Fig. 3.1) was retrieved in association with rainbow trout *Onchorynchus mykiss* and is the putative agent of strawberry disease/red-mark syndrome in the fish (Lloyd et al. 2008). Details on this organism are provided in a separate chapter (Chap. 14). Other sequences were retrieved in association with the marine sponge *Haliclona* sp. from California, USA (Sipkema et al. 2009) and to the rhizarian *Reticulomyxa filosa* (Glöckner et al. 2014). Thus, it appears likely that a number of new genus-level lineages of "*Ca*. Midichloriaceae" could be characterized and described in the next future.

5 Rickettsiales Incertae Sedis

A *Rickettsiales* bacterium, termed by the authors as **R1 symbiont**, was found in association with the phagotrophic euglenid *Petalomonas sphagnophila* from Canada (Kim et al. 2010). According to the report by Kim and coauthors, the R1 symbiont was described as a rod-shaped Gram-negative bacterium, 0.5 μm long and 0.15 μm wide, enclosed by a presumably host-derived membrane layer (Kim et al. 2010). Other bacteria were found in coexistence within the same protist host, namely a "*Ca*. Captivus acidiprotistae"-related bacterium, a *Coxiella*-related bacterium, and representatives of *Firmicutes* and *Deltaproteobacteria*. The phylogenetic position

of the R1 symbiont does not appear fully resolved. According to the authors' analysis, it belongs to a lowly supported sister-clade of *Rickettsiaceae* (Kim et al. 2010). Considering also the relatively low similarities with representatives of the three *Rickettsiales* families (≤87 %, very close to the 86.5 % family threshold by Yarza et al. 2014), this could represent a fourth family level lineage. Further dedicated studies will be necessary to clarify this point.

6 General Conclusions and Perspectives

As described in detail in this chapter, in the last two decades the diversity of *Rickettsiales* and their eukaryotic hosts are revealed to be much wider than previously recognized. The current knowledge on *Rickettsiales* diversity can be summarized in the following points:

- Together with the two "traditional" families of *Rickettsiales*, namely *Rickettsiaceae* and *Anaplasmataceae*, a third one, "*Ca*. Midichloriaceae" was described (Montagna et al. 2013). The diversity of "*Ca*. Midichloriaceae" is comparable to the other two families.
- Currently, 24 genera of *Rickettsiales* are recognized (eight *Rickettsiaceae*, seven *Anaplasmataceae*, nine "*Ca*. Midichloriaceae"), out of which six are "traditional" genera and 18 were described since year 2000.
- The majority (16 out of 24) of currently known genera include (and in most cases are completely constituted by) representatives found in association with aquatic organisms, in several cases protists.

The phylogenetic relationships of *Rickettsiales* hosted by terrestrial organisms (such as ticks and other arthropods) and those hosted by aquatic organisms (such as ciliates, amoebae, algae, and cnidarians) are highly interwoven (Fig. 3.1), implying that these bacterial endosymbionts were repeatedly able to switch from one host to another. Thus, although phylogenetic inference alone cannot provide a final answer on this point, it seems probable that the ancestral hosts of *Rickettsiales* were aquatic organisms, possibly protists, as already suggested by some studies (Vannini et al. 2005, 2010; Ogata et al. 2006; Weinert et al. 2009; Schrallhammer et al. 2013). Moreover, it seems important to underline that the adaptations to terrestrial arthropods should then have occurred several times independently, namely at least twice within each of the three families (Fig. 3.1).

Therefore, it appears evident that the study on diversity of such "non-model" *Rickettsiales*, in particular those associated to aquatic organisms, is necessary to get a complete perspective on the evolutionary history of the whole group. It can also offer some insight on the origin of mitochondria since most studies consider the bacterial ancestor of these organelles phylogenetically closely related to *Rickettsiales* (Gray et al. 1999; Fitzpatrick et al. 2006; Rodríguez-Ezpeleta and Embley 2012).

The relationships with the eukaryotic hosts of "non-model" *Rickettsiales* have not been extensively studied and, up to now, there is no full evidence indicating that

some of them can be pathogenic for humans or other vertebrates although a possibility of transmission and/or a linkage to pathogenic action was found in some cases (see Chap. 14). Nevertheless, considering the interwoven relationships with medically relevant relatives, "non-model" *Rickettsiales* can become important models for a better understanding of the different mechanisms of interaction with eukaryotic cells and their evolutionary origin, in particular those involved in pathogenesis. Indeed, some relevant features have been already evidenced:

- **Flagella** were observed in electron microscopy and/or **flagellar genes** were found in the genomes of **nine *Rickettsiales*** (four among *Rickettsiaceae*, namely "*Ca*. Trichorickettsia," "*Ca*. Gigarickettsia," "*Occidentia*," and "*Ca*. Arcanobacter"; one among *Anaplasmataceae*, namely "*Ca*. Xenolissoclinum"; four among "*Ca*. Midichloriaceae", namely "*Ca*. Midichloria," *Lyticum*, "*Ca*. Bandiella," and "*Ca*. Jidaibacter"). The phylogenetic inference of flagellar genes is consistent with vertical inheritance from the common ancestor of *Rickettsiales* (Sassera et al. 2011), suggesting repeated independent flagellar gene losses in the other extant lineages of the order. Given the obligate intracellular lifestyle of *Rickettsiales*, the function of flagella is not obvious. For those endosymbionts retrieved from aquatic environments, a reasonable hypothesis is that these motile structures could be helpful for transmission among different hosts (Schulz et al. 2016). This possibility is also consistent with the retrieval in "*Ca*. Arcanobacter lacustris" of genes for **bacterial chemotaxis**, which could work in synergy with flagella to direct bacterial movement. On the other hand, "*Ca*. Midichloria mitochondrii" and "*Occidentia massiliensis*" could represent intermediate cases between aquatic *Rickettsiales* displaying flagella/flagellar genes and terrestrial ones which have lost those genes, due to a more recent adaptation to terrestrial arthropod hosts. Flagellar components could also be involved in other functions, in particular in delivering effectors to the host considering the homology with type III secretion systems (Sassera et al. 2011; Mariconti et al. 2012b).
- Components of **type IV secretion systems** were found. In particular the **VirB** system, which is typical in medically relevant *Rickettsiales* and is thought to be involved in pathogenesis (Gillespie et al. 2010, 2015), was found in all the **six** "**non-model**" ***Rickettsiales*** for which at least partial genomic or transcriptomic data is available (two among *Rickettsiaceae*, namely "*Ca*. Megaira" and "*Ca*. Arcanobacter"; one among *Anaplasmataceae*, namely "*Ca*. Xenolissoclinum"; three among "*Ca*. Midichloriaceae", namely "*Ca*. Midichloria", "*Ca*. Jidaibacter" and "*Ca*. Bandiella"). In the same organisms (with possible exception "*Ca*. Bandiella," for which however only few partial gene sequences are available) **putative effectors** were found as well, such as proteins including **ankyrin**, **leucine-rich**, or **tetratricopeptide repeats**, which are rare in prokaryotes. In *Rickettsiales* (and other intracellular bacteria), they are thought to be involved in interaction with the eukaryotic host (Gillespie et al. 2015), in particular influencing gene expression (McBride and Walker 2011). These findings are consistent with the hypothesis that the role of VirB type IV secretion systems was ancestral in *Rickettsiales* (Gillespie et al. 2010).

- The homologs of the **rOmpB** of *Rickettsia* and its interactor **Ku70** from the host were found, respectively, in "***Ca***. **Megaira polyxenophila**" and its unicellular non-phagocytic host *M. viride*. In *Rickettsia*, this system is implicated in induction of the phagocytotis for invasion of target host cells such as those of endothelium. Thus, taking into account the relatively close relationship of *Rickettsia* and "*Ca*. Megaira" and the above hypothesized ancestrality of aquatic protist hosts for *Rickettsiales*, this finding suggests a possible evolutionary route for the origin of the system in the sublineage of *Rickettsiaceae* including these organisms.
- The ***Rickettsiaceae*** endosymbionts "*Ca*. Trichorickettsia mobilis" and "*Ca*. Megaira polyxenophila" were found in the **macronucleus** of some ciliate host strains. Considering that in few cases representatives of genus *Rickettsia* and *Orientia tsutsugamushi* were found as well in the nucleus of host cells (Wolbach 1919; Burgdorfer et al. 1968; Urakami et al. 1982; Watanabe et al. 2014), it is possible to speculate that the ability of entering host nuclear apparatus was ancestral at least in the *Rickettsiaceae* lineage.

In conclusion, it should also be underlined that the current knowledge on the diversity of *Rickettsiales* lineages is certainly still incomplete, so that new genera or even families could be discovered in the future. This point is convincingly evidenced by several studies, for example, the discovery of the R1 endosymbiont of *P. sphagnophila*, which possibly belongs to a new family of *Rickettsiales* (Kim et al. 2010). In addition, the retrieval of several short 16S rRNA gene sequences corresponding to uncharacterized organisms with affinity to *Rickettsiales* in public archives resulting from high-throughput sequencing, such as evidenced in the study by Martijn and coauthors (Martijn et al. 2015), supports this view.

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Part II
Rickettsiales: Diagnosis and Management of Diseases

Chapter 4
Rickettsiales: Laboratory Diagnosis

Lucas S. Blanton

1 Introduction

The order *Rickettsiales* is composed of two diverse families of organisms (*Rickettsiaceae* and *Anaplasmataceae*), which cause a variety of different illnesses distributed throughout the world. In the last two decades, there has been a marked increase in the discovery of novel species (Walker et al. 2008). For example, the spotted fever group (SFG) of the genus *Rickettsia* has over 20 named species and species candidates. While many of these are associated with human disease, others have been discovered in arthropods with little knowledge of their potential pathogenicity in humans. Even those thought to be nonpathogenic at one point in time have later been implicated as a cause of human disease (i.e., *Rickettsia parkeri*) (Paddock et al. 2004). If keeping up with the large and growing list of emerging pathogens in this order is not challenging enough for clinicians, the relatively undifferentiated and protean clinical manifestations add a tremendous amount of complexity to the recognition of these infections. Furthermore, with globalization and ease of travel, it is increasingly possible for patients to be exposed to rickettsial pathogens endemic to other parts of the world (Blanton 2013). Fortunately, many aspects regarding diagnostic principles are similar among the various clinical syndromes caused by the *Rickettsiales*. Although serology is the mainstay of diagnostic methods, techniques using polymerase chain reaction, immunohistochemistry, and culture offer alternative methods to help establish a diagnosis. This chapter will outline the approach to diagnosing patients with suspected infection caused by organisms in the genus *Rickettsia*, *Orientia*, *Ehrlichia*, and *Anaplasma*.

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2 General Principles

One of the most important aspects of diagnosing infections caused by organisms in the order *Rickettsiales* is knowledge and recognition of their clinical syndromes. Unfortunately, because of their undifferentiated nature, this is often difficult. The spectrum of etiologic causes for such a syndrome is diverse and may include a variety of relatively common viral syndromes (e.g., influenza, enterovirus, Epstein–Barr virus, and cytomegalovirus). Furthermore, in tropical regions of the world, the signs and symptoms of these illnesses can mimic a variety of other febrile syndromes (e.g., malaria, leptospirosis, dengue fever, and other arboviral infections). Although clues such as an eschar or rash may help a clinician include infections caused by *Rickettsiales* in their differential diagnosis, these clues are often subtle or may be absent (Fig. 4.1). Even when present, these signs are not specific for a rickettsial illness. For example, the differential diagnosis for a patient with an ulcerative or eschar-like lesion may include cutaneous anthrax, plague, tularemia, syphilitic chancre, and leishmaniasis.

Although often indistinguishable from other causes of fever, inclusion of a rickettsial illness in the differential diagnosis can stimulate historical questions to help a clinician sort through the long list of etiologic possibilities. Simply asking a patient about recent tick bites or seeing ticks may be helpful, but since larval and nymphal stages are small and often go unnoticed, denial of tick bites should not preclude the possibility of a rickettsial infection. Questions pertaining to arthropod exposure through occupational or recreational activities (e.g., hiking, camping, hunting) may be helpful. A travel history should be obtained when approaching such a patient (Jensenius et al. 2004). African tick bite fever is a frequent cause of febrile illness in travelers returning from Sub Saharan Africa (Jensenius et al. 2003). Those with the illness are often exposed to aggressive *Amblyomma* ticks while on safari (Fig. 4.2). There have also been several reports of murine typhus being acquired during travel (Walter et al. 2012). Although exposure to many of these

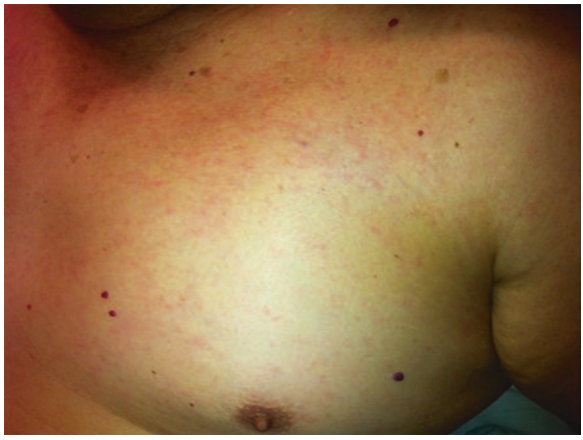

Fig. 4.1 The rash in a patient with murine typhus. This faint macular rash was not present during the patient's first few days of illness. He was empirically treated with doxycycline and made a speedy recovery. Although serology during acute illness was nonreactive for typhus group antibodies, he had a titer of 1:2048 several weeks after convalescence

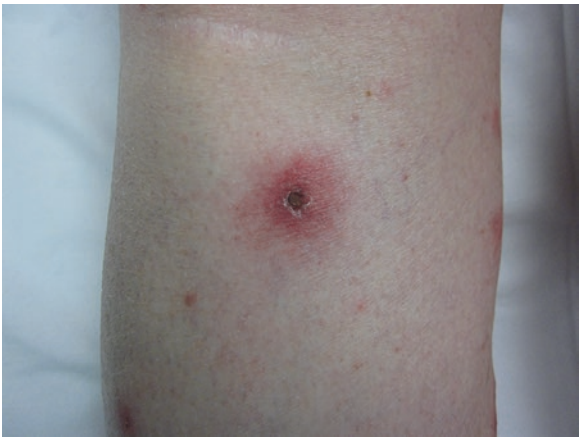

Fig. 4.2 An eschar in a woman with African tick bite fever. The woman traveled to Kruger National Park in South Africa. She reported numerous tick bites while on Safari

pathogens is often thought to be of rural origin, it should be noted that the flea-borne rickettsiosis murine typhus, caused by *R. typhi*, is associated with reservoir-vector cycles of transmission that are both urban (rats and the oriental rat flea) and suburban (opossums and the cat flea) (Civen and Ngo 2008). Furthermore, urban parks may serve as a habitat for ticks infected with rickettsiae and ehrlichiae (Blanton et al. 2014; Salgo et al. 1988).

An important patient management point regarding these diseases is prompt empiric antimicrobial administration when any of these infections are suspected (Chapman et al. 2006). Currently, there is no available rapid test with adequate sensitivity to rule out infection from any of these organisms. Current diagnostic techniques are either not sensitive enough in acute illness (PCR from peripheral blood), not widely available (immunohistochemistry), or retrospective in nature (serology). Because some of these illnesses can be quite severe with a high untreated case fatality (e.g., Rocky Mountain spotted fever (RMSF)), appropriate antimicrobial therapy should not wait for a confirmation of diagnosis.

3 Culture

As obligately intracellular organisms that require host cells to survive, axenic growth has not been accomplished on cell-free media. The laboratory isolation of these bacteria require inoculation onto living cells such as the yolk sac of embryonated eggs, experimental animals, or cultured cell lines. Isolation from humans can be made using blood, buffy coat, or tissue inoculated on the appropriate cell line. Culture is rarely undertaken as a means of diagnosis for several reasons—isolation requires technical expertise, maintenance of cell lines, and specialized laboratory facilities (La Scola and Raoult 1997). Organisms in the genus *Rickettsia* and *Orientia* are small and easily aerosolized, which necessitates the use of a biosafety

level 3 laboratory to carry out these techniques. The shell vial technique of isolation for organisms in the genus *Rickettsia* is effective and can yield results in a relatively short period of time when the inoculated cell culture monolayer is later fixed and examined microscopically using immunofluorescent techniques to visualize rickettsiae (Marrero and Raoult 1989; La Scola and Raoult 1996). Culture of *Ehrlichia* spp. and *Anaplasma phagocytophilum* can be undertaken in a biosafety level 2 laboratory (Standaert et al. 2000).

4 Serology

4.1 Spotted Fever and Typhus Group Rickettsioses

Serologic methods are the most widely used diagnostic tools for the diagnosis of SFG and typhus group rickettsioses. The gold standard method is the indirect immunofluorescence assay (IFA), which uses a fluorescent-labeled conjugate to detect antibodies bound to rickettsial antigens fixed on a slide. This method is superior to older techniques (e.g., Weil-Felix, latex agglutination, complement fixation) (Kaplan and Schonberger 1986; Eremeeva et al. 1994a, b). IFA for the diagnosis of spotted fever and typhus group infections are available commercially and are also available through state health department laboratories. In the United States, whole cell *R. rickettsii* and *R. typhi* antigens are often used for the diagnosis of spotted fever group and typhus group rickettsioses, respectively.

Infection from a rickettsial species of a certain group will induce antibodies that cross-react with antigens from different species of the same group. For example, the serum of a patient infected with the SFG pathogen *R. parkeri* will react if tested against *R. rickettsii*. Although antibody titers are usually higher when reacted against homologous antigen compared to heterologous antigen, determining a species-specific cause of illness is generally not possible via standard serologic testing. In the case of the typhus group, where only two species exist (*R. typhi* and *R. prowazekii*), knowledge of the local epidemiology can help a clinician make a distinction between endemic and epidemic typhus when a patient's serum is reactive using methods to detect typhus group antibodies. Unfortunately, the numerous and growing list of species in the SFG makes this distinction impossible—in the Southeast United States alone, ticks harbor a number of pathogenic and nonpathogenic species (e.g., *R. rickettsii*, *R. parkeri*, *R. montanensis*, *R. amblyommii*), which can each induce antibodies cross-reactive against antigen derived from other SFG rickettsiae. Because of the diversity of the SFG and the varying geographic distribution of its member species, it is important that laboratories use antigen prepared from rickettsial species that cause infections that are endemic to the region of interest. In areas where there exists the possibility of several endemic rickettsioses, the micromethod format of the IFA (micro-IFA) can test serum against up to nine different antigens, as it uses multiple antigen dots placed on a single well (La Scola and Raoult 1997).

The development of antibodies occurs relatively late in the course of illness for SFG and typhus group infections. Usually, there are no detectable antibodies in the first several days of illness. By the second week, following the onset of symptoms, almost all patients will have detectable antibodies by IFA (Chapman et al. 2006; Dumler et al. 1991). Both the IgM and IgG isotypes appear at relatively the same time during a patient's course (Clements et al. 1983); therefore, testing for IgM does not increase the sensitivity of IFA at early time points as compared to testing for IgG. Furthermore, IgM antibodies have a predilection to cross-react with nonspecific antigens more often than the IgG isotype, which limits the specificity of IgM assays. Anti-SFG IgM cross-reacts nonspecifically with the lipopolysaccharide of *Proteus* and *Legionella* species (Raoult and Dasch 1995). Among patients being worked up for RMSF, 46 % of those with a reactive IgM at an early time point failed to develop a subsequent reaction with IgG. These reactive IgM assays therefore represented erroneous results (McQuiston et al. 2014). Finally, IFA testing for IgM can also be affected by circulating rheumatoid factor, so a rheumatoid factor absorbant must be used prior to incubation of sera on antigen slides.

Since the antibody response lags several days behind the onset of the initial clinical syndrome, a nonreactive IFA should not rule out the possibility of an SFG or typhus group rickettsiosis. Serum for serologic testing should be obtained during acute illness as well as after recovery. Either seroconversion or a fourfold rise in titers from acute- to convalescent-phase sera confirms the diagnosis of a rickettsial illness. A single titer of at least 1:256 obtained during a compatible illness is highly suggestive of the diagnosis (Chapman et al. 2006).

Generally speaking, the principle behind IFA for the diagnosis of the SFG and typhus groups is similar. It should be noted that sera reactive against SFG antigen at high titers may be reactive against typhus group antigen at much lower titers. The same holds true for sera reactive against typhus group antigen at high titers. This is due to cross-reactive non-LPS antigens common to organisms in both rickettsial groups. Another difference regarding the reading of IFA slides between the SFG and typhus groups is related to their growth in cells. Unlike organisms in the SFG, organisms in the typhus group (i.e., *R. typhi* and *R. prowazekii*) tend to burst cells while being propagated in cell culture. Therefore, IFA slides using these two antigens may show many extracellular forms. The fluorescence of these forms resembles a fine gold dust-like appearance. This may be mistaken as artifact or background, making these slides more difficult to read than slides prepared for the diagnosis of SFG rickettsioses or scrub typhus (Phetsouvanh et al. 2013).

Besides the IFA, the enzyme-linked immunosorbent assay (ELISA) is commercially available for the diagnosis of rickettsioses. These methods are sensitive, easily automated, and lend themselves to efficiency, as they offer laboratories a high throughput method for anti-rickettsial serologic testing. This method also uses a plate reader to interpret results. Therefore, the need for a skilled microscopist and the subjectivity of reading IFA slides is avoided. Of course when whole cell rickettsial antigen is used on IFA slides, and when a proficient microscopist is available, the visualization of intracellular forms with morphology resembling *Rickettsia* enhances the specificity of IFA over that of ELISA, as the latter is prone to a higher

false positive rate. Another drawback of the ELISA includes the inability to accurately quantify antibody titers. This limits its ability to follow the serologic response over the course of time (Chapman et al. 2006).

4.2 Scrub Typhus

The gold standard serologic method for the diagnosis of infection caused by *Orientia tsutsugamushi* is the IFA. The sensitivity and specificity is excellent, but like the SFG and typhus group rickettsioses mentioned above, antibodies are often not present during early illness (Bozeman and Elisberg 1963). Another technique similar to that of IFA is the immunoperoxidase assay (IPA). The performance of the IPA is similar to that of the IFA, but the method holds a few advantages that make it attractive in resource limited settings that are endemic for scrub typhus. The IPA uses a light microscope rather than a fluorescent one and is less costly in regard to reagents (Yamamoto and Minamishima 1982; Kelly et al. 1988). The determination of endpoint titers using these two techniques is somewhat subjective. With either method, experience is important for the accurate reading of slides, as there is greater interoperator variability among microscopists with less experience (Phetsouvanh et al. 2013).

One difficulty regarding the serodiagnosis of scrub typhus involves the tremendous antigenic variation of *O. tsutsugamushi*—there are over 70 known strains (Paris et al. 2013). There is variable cross-reactivity among these varying strains, so use of antigen derived from strains endemic to one area may not be optimal for diagnostics in another area. For this reason, the three prototype *O. tsutsugamushi* strains (Karp, Kato, and Gilliam) are often used in conjunction to regional strains during the preparation of antigen for IFA and IPA.

Confirmation of diagnosis can be accomplished by demonstrating seroconversion or a fourfold increase in endpoint titers between serum samples obtained during the acute- and convalescent-phase of illness. Since reinfection can occur after immunity wanes, the seroprevalence may be high in endemic areas. Because of this, there is no consensus regarding the optimum cutoff titer. In areas where scrub typhus is highly endemic, cutoff titers as high as 1:400 have been proposed (Blacksell et al. 2007).

4.3 Ehrlichiosis and Anaplasmosis

Both *E. chaffeensis* and *A. phagocytophilum* can be directly observed on Wright-stained peripheral blood or buffy coat smears, but this method is not adequately sensitivity, especially for the diagnosis of HME. Smears have been found to be positive in 1–20 % of those with HME (Paddock and Childs 2003; Chapman et al. 2006) and 61–80 % in those with HGA (Bakken et al. 1996, 2001). Therefore, as similar

to the aforementioned syndromes, serology is the mainstay of diagnosis for those with suspected ehrlichiosis or anaplasmosis. The IFA is the serodiagnostic method of choice. Slides prepared with cells infected with *E. chaffeensis* and *A. phagocytophilum* are used to diagnose human monocytotropic ehrlichiosis (HME) and human granulocytotropic anaplasmosis (HGA), respectively. When incubated with reactive sera, IFA slides reveal the characteristic mulberry-like inclusions of bacteria (morula) within the cytoplasm of the fixed cells. Those living in highly endemic areas for HME and HGA may have baseline serologic reactivity related to previous exposure to the pathogens responsible for these illnesses (Aguero-Rosenfeld et al. 2002). It is therefore necessary to confirm the diagnosis with a fourfold rise in antibody titers from sera obtained during acute illness and after convalescence.

The use of paired sera to confirm the diagnosis of HME or HGA is also important as few patients will have detectable antibodies during the first days of illness. During the first week of illness only 22–44 % of patients with HME will have a reactive titer (Dawson et al. 1990), while 24–44 % of those with HGA will have a reactive titer in their first week of illness (Walls et al. 1999; Bakken et al. 2002). After a week's time, titers generally increase. In the case of HME, 68 % of patient sera will yield a reactive titer after the seventh day. Negative serology should therefore not be used to exclude the diagnosis or withhold effective antibiotic therapy. By the fourth week of illness, all patients with HME or HGA will have seroconverted (Dawson et al. 1990; Bakken et al. 2002).

5 Immunohistochemistry

Prior to molecular methods, immunohistochemistry (IHC) was the only diagnostic method that could accurately establish the diagnosis of a rickettsiosis during the acute presentation of illness. The technique involves the staining of organisms within formalin-fixed paraffin-embedded tissues prior to the hosts development of anti-rickettsial antibodies (Walker et al. 1978). In those with RMSF, skin biopsy specimens of the rash have a reported sensitivity and specificity of 70 % and 100 %, respectively (Walker 1995). Of the eschar-associated SFG rickettsiae (e.g., *R. conorii*, *R. parkeri*, *R. africae*), IHC can establish the diagnosis by visualization of organisms within a biopsied eschar. Autopsy specimens such as tissue from the liver, spleen, lung, heart, kidney, and brain can be tested using IHC. These techniques have also been applied to typhus group rickettsioses. Successful use of this technique has been reported using tissues from autopsy specimens of those with epidemic typhus and murine typhus (Walker et al. 1989, 1997). Use of skin biopsy specimens has also been reported in murine typhus (Blanton et al. 2015).

Immunohistochemical visualization of morulae from tissue is also an effective technique for the diagnosis of HME and HGA. The lung, liver, spleen, lymph nodes, kidneys, adrenal glands, and bone marrow have demonstrated organisms when collected at the time of autopsy (Dumler et al. 1993; Dawson et al. 2001). Since HME and HGA are often accompanied by cytopenias, bone marrow biopsy specimens

may be frequently obtained as part of a patient's clinical workup. Although IHC offers the chance to make a diagnosis prior to the production of detectable antibodies by the host, it is important to note that IHC is not as readily available as other methods for the diagnosis of these infections. In addition, accurate reading and interpretation of immunostained tissues requires special skill and expertise.

6 Molecular Detection

6.1 Spotted Fever and Typhus Group Rickettsioses

Polymerase chain reaction (PCR) is a method that may be used to detect rickettsial DNA from a variety of clinical samples. Prior to the advent of molecular techniques, IHC was the only method to establish confirmation of diagnosis during the early course of illness. PCR methods used for the detection of rickettsial DNA include conventional, nested, and real-time assays. Unfortunately, the easiest sample to obtain, blood, yields poor sensitivity for the amplification of SFG or typhus group rickettsioses. As intracellular organisms with an endothelial tropism, the organism does not seem to be persistently circulating at detectable levels in the bloodstream. This is especially true during mild illness, whereas detectable rickettsemia seems to occur more often in severe or fatal cases (Tzianabos et al. 1989; Sexton et al. 1994). Although whole blood testing lacks sensitivity, the PCR amplification of rickettsial DNA from biopsied tissue specimens (e.g., skin biopsy of a rash lesion) is of much greater yield. In eschar-associated rickettsioses, an eschar biopsy is an excellent site for PCR testing (La Scola and Raoult 1997). An eschar swab is a less invasive method of obtaining material for PCR testing. The technique involves the vigorous swabbing of an unroofed eschar with a saline moistened sterile cotton tipped swab. The collected swab is then placed in tube with a small amount of saline to be processed (Mouffok et al. 2011). The chance of successful amplification of rickettsial DNA by PCR is best prior to antimicrobial treatment (Chapman et al. 2006).

A variety of different primers have been developed for the detection of rickettsial DNA. These are generally genus-specific and amplify portions of conserved rickettsial genes—citrate synthase, outer membrane protein B (OmpB), or the 17-kDa antigen gene (Tzianabos et al. 1989; Regnery et al. 1991; Roux and Raoult 2000). Unlike SFG rickettsieae, those in the typhus group lack the outer membrane protein A (OmpA) (Fournier et al. 1998). Therefore, amplification of a portion of the OmpA gene would exclude a typhus group organism as the culprit. Qualitative real-time PCR seems to offer greater sensitivity compared to conventional and nested assays (Kato et al. 2013).

Since treatment is similar for all these illnesses, establishing a species-specific diagnosis for the purposes of individual patient care is usually not sought, but identification of an offending rickettsial agent on a species level may be sought for the purposes of clinical or epidemiological research. The primary method for establishing a specific diagnosis is through nucleotide sequencing of amplified rickettsial PCR

products. Because many of targeted rickettsial genes are relatively conserved, it may be necessary to amplify and sequence portions of many genes, large portions of genes, or sequence genes with more diversity among species (e.g., OmpA for the spotted fever group). Restriction fragment length polymorphism (RFLP) analysis is another method that can be used to delineate one rickettsial species from another. Several methods have been reported using a variety of endonucleases for amplified products of different genes (Boostrom et al. 2002; Eremeeva et al. 1994a; Regnery et al. 1991). One such method uses several endonucleases (*Rsa*I, *Pst*I, *Alu*I, *Xba*I, and *Ava*II) on an amplified portion of *OmpA* to identify most of the recognized SFG pathogens (Roux et al. 1996). Although RFLP analysis is relatively inexpensive, the price of sequencing has dropped dramatically. The use of nucleotide sequencing provides much more information, especially if there is concern for a novel strain or species.

6.2 Scrub Typhus

The DNA of *O. tsutsugamushi* can be detected in a variety of clinical specimens by PCR. Conventional, nested, and real-time PCR assays have all been successfully used. Protocols using primers to amplify the 56-kDa protein gene, 47-kDa protein gene, *groEL*, and the 16S rRNA gene have been developed. A variety of clinical specimens can be tested by molecular methods and include whole blood, buffy coat, eschar punch biopsies, and eschar crust. In one center, conventional PCR of buffy coat specimens directed at a 16s RNA target was 87 % sensitive and 100 % specific (Kim et al. 2016). A nested PCR reaction to amplify a portion of the 56-kDa gene from eschar specimens has been reported to be 86 % sensitive and 100 % specific when compared to IFA as the gold standard (Kim et al. 2006). Quantitative real-time PCR assays seem to offer excellent sensitivity as well—they have the ability to detect low copy numbers of targeted *O. tsutsugamushi* genes (Jiang et al. 2004; Paris et al. 2009).

6.3 Ehrlichiosis and Anaplasmosis

The tropism of *E. chaffeensis* for monocytes makes PCR a much more useful tool for the diagnosis of HME than for SFG or typhus group rickettsioses when applied to blood or buffy coat samples. The pathogen can also be detected from cerebrospinal fluid (Dunn et al. 1992), tissue samples (Paddock et al. 1997), and serum—the latter has a much lower yield (Massung et al. 1998). Methods to detect ehrlichial DNA include conventional, nested, and real-time PCR assays (Massung et al. 1998; Childs et al. 1999; Loftis et al. 2003). Qualitative real-time PCR is very sensitive and can detect as few as one gene copy per PCR reaction (Loftis et al. 2003). A multiplexed PCR assay using species-specific primers and probes for the *Ehrlichia*-specific disulfide bond formation gene can differentiate between both *E. chaffeensis* and *E. ewingii* (Doyle et al. 2005).

As in the case of infection with HME, those with HGA have organisms circulating in the peripheral blood within neutrophils (Edelman and Dumler 1996; Horowitz et al. 1998). The yield of PCR for the diagnosis of HGA is greatest during early illness, prior to the induction of anti-*Anaplasma* antibodies (Schotthoefer et al. 2013). PCR assays targeting genes with multiple copies within the bacterial genome are reported to be more sensitive than assays with only one gene target per organism (Wang et al. 2004). Molecular methods have been paramount for the discovery of new agents. The *Ehrlichia muris*-like agent was discovered after melt curve analysis of a real-time PCR assay targeting the *groEL* gene was found to be inconsistent with that of the targeted pathogens (Pritt et al. 2011). Specific PCR assays have now been developed for the diagnosis of infection with this agent (Allerdice et al. 2016).

7 Summary

Diseases caused by *Rickettsia*, *Orientia*, *Ehrlichia*, and *Anaplasma* are protean and undifferentiated in regard to their signs and symptoms. They are therefore extremely hard to diagnose. Clinical suspicion is the key to the initiation of timely therapy and clinical workup. Serology is the mainstay of diagnostic methods, but since antibody production occurs later in illness, diagnosis is usually retrospective and requires seroconversion or a fourfold increase in titers from acute- and convalescent-phase sera. Immunohistochemistry offers a method of diagnosis during acute illness, but few physicians will have access to this method. Molecular methods also offer the chance of making a diagnosis during early illness, but the sensitivity of these assays is dependent on the pathogenesis of the infecting organism and the site of DNA acquisition (e.g., blood, buffy coat, tissue).

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Chapter 5
Rickettsiales: Treatment and Management of Human Disease

Lucas S. Blanton

1 Introduction

Syndromes associated with organisms in the order *Rickettsiales* range from mild illness, which may fail to prompt the seeking of medical attention, to severe life-threatening infections. Infections with these agents are not limited to those with severe medical comorbidities or immunocompromised conditions; rather, anyone exposed to potential vectors (even the young, active, and healthy) is at risk for acquiring an infection. Fortunately, if the possibility of a rickettsiosis is promptly recognized, the prompt initiation of an effective antibiotic can prevent morbidity and mortality (Hamburg et al. 2008), especially in the setting of a severe disease such as Rocky Mountain spotted fever (RMSF). Effective therapy is readily available and generally inexpensive, but many empiric antibiotic choices such as beta-lactams or sulfonamides, targeting more frequently occurring syndromes (e.g., pharyngitis, upper respiratory tract infections, urinary tract infections), have no activity against these rickettsial pathogens (Rolain et al. 1998; Branger et al. 2004). This chapter is intended to guide the reader on appropriate pharmacologic therapy for those infected with organisms in the order *Rickettsiales*. The following sections will outline the general principles when approaching a patient with a suspected rickettsiosis; discuss important pharmacologic properties of active antimicrobial agents; discuss specifics regarding important clinical syndromes; and finally, touch upon some important aspects regarding the treatment of children and pregnant women.

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2 General Principles

As largely undifferentiated febrile illnesses with signs and symptoms mimicking many other infectious diseases, it is imperative for clinicians to be aware of the specific rickettsial agents endemic to their region of practice and to recognize the potential for acquiring these infections while traveling (Parola et al. 2013). The patient's history in regard to occupation, recreational activities, and exposure to potential arthropod vectors and mammalian reservoirs is paramount in formulating the differential diagnosis in a patient with a fever without an obvious source. Although rash is often the sign that clues a physician to include a rickettsial illness as a consideration, the presence of a typical rash varies depending on the infecting agent. For example, rash occurs in 90 % with RMSF (*R. rickettsii*) (Helmick et al. 1984) versus 54 % with murine typhus (*R. typhi*) (Dumler et al. 1991). Rash may occur late in the course of illness (50 % after day 3 in those with RMSF) (Helmick et al. 1984). Furthermore, rash is a less frequent occurrence in human monocytotropic ehrlichiosis (HME) (Fishbein et al. 1994; Olano et al. 2003) and human granulocytotropic anaplasmosis (HGA) (Aguero-Rosenfeld et al. 1996; Bakken and Dumler 2008). Since delay of treatment can have severe consequences and portend a higher case fatality rate (Kirkland et al. 1995), clinicians should not rely on the presence of rash when considering empiric treatment. Subtle physical exam findings, such as an eschar, may be hidden and require close examination of normally clothed areas of the skin (genitalia, buttock, or axilla), but their presence may be a strong indicator of the site of bacterial inoculation. In those with darkly pigmented skin, a subtle rash may not be easily noticed, as it may be in a patient with lightly pigmented skin.

When infection with these agents is suspected, prompt empiric antimicrobial therapy should be started. Confirmatory laboratory diagnosis with serology, the mainstay of diagnostic techniques, is retrospective in nature, requiring seroconversion or fourfold increase in antibody titers from acute- and convalescent-phase serum samples. Therefore, antimicrobials should not be withheld while awaiting confirmation of diagnosis (Chapman et al. 2006).

The antibiotics used to treat these infections are relatively bioavailable by the oral route, facilitating outpatient management—provided the patient is able to tolerate oral medications and has access to appropriate follow-up. Since nausea and vomiting often accompany severe disease, admission to the hospital for initiation of parenteral antibiotics may be necessary. Other indications for hospitalization, and perhaps admission into an intensive care unit, may include the need for close clinical monitoring, management of hemodynamic instability with intravenous fluids and/or vasopressors, and other supportive measures (e.g., ventilatory management and hemodialysis). The increased vascular permeability associated with severe infections may complicate the balance of maintaining end organ perfusion through maintenance of the intravascular fluid compartment versus extravasation of fluid into the interstitial third space. For the sake of optimal ventilatory support, this may necessitate the need for Swan Ganz catheterization to monitor pulmonary capillary wedge pressure.

3 Antimicrobial Agents

Many antimicrobials frequently used for the empiric treatment of a presumed bacterial infection are ineffective for the treatment of rickettsioses (e.g., penicillins, cephalosporins, sulfonamides, and aminoglycosides) (Rolain et al. 1998; Branger et al. 2004). Sulfonamide formulations, such as trimethoprim-sulfamethoxazole, have even been associated with increased severity and poorer outcomes (Ruiz Beltran and Herrero Herrero 1992a). For the most part, treatment for these illnesses is fairly similar and lean on the tetracycline antibiotic class as the treatment of choice. For the exception of prospective studies performed in those with Mediterranean spotted fever (MSF), most treatment recommendations are based on retrospective data supporting the efficacy of tetracyclines. Susceptibilities for antibiotics against rickettsial agents are not standardized or validated in the same manner as they are for more typical bacterial agents. The following section will highlight important aspects of antimicrobial agents with in vitro activity to clinically relevant rickettsioses. Although not meant to be as extensive in detail, nor substitute for a pharmacologic text, information will include usual dosages, potential side effects, and contraindications. When available, the MICs for drugs against these pathogens are mentioned. Following sections will touch upon caveats of therapy regarding specific disease syndromes and in special circumstances.

3.1 Tetracyclines

The tetracyclines are the treatment of choice for all agents in the order *Rickettsiales*. This class includes tetracycline hydrochloride, doxycycline, and minocycline. Tetracycline hydrochloride is a short half-life drug, which is given four times daily. The presence of food inhibits its absorption. As a consequence of having to take on an empty stomach, gastrointestinal side effects are fairly common. Although inexpensive and readily available throughout the world, the drug is not widely available in the United States. Doxycycline and minocycline have good bioavailability with food. In fact, the presence of food in the stomach can alleviate the potential side effects of nausea and dyspepsia. The longer half-lives of doxycycline and minocycline allow for twice daily dosing, another factor that improves adherence. These medications should be taken with ample water to ensure passage through the gastroesophageal junction and to avoid pill esophagitis (Moffa and Brook 2015).

The in vitro susceptibility of tetracyclines to spotted fever group rickettsiae (SFGR), typhus group rickettsiae (TGR), *Orientia tsutsugamushi*, *Ehrlichia chaffeensis*, and *Anaplasma phagocytophilum* are excellent and appear better than that of antibiotics in other classes (McDade 1969). The MICs of the SFGR and TGR to tetracyclines are 0.06–0.25 μg/mL (Rolain et al. 1998); those for *Orientia tsutsugamushi* are 0.15–0.31 μg/mL (Raoult and Drancourt 1991); those for *Ehrlichia chaffeensis* are <0.5 μg/mL (Brouqui and Raoult 1992; Branger et al.

2004); and those for *Anaplasma phagocytophilum* are 0.03–0.25 μg/mL (Klein et al. 1997; Horowitz et al. 2001; Maurin et al. 2003; Branger et al. 2004). The standard dose of doxycycline and minocycline in adults is 100 mg oral or intravenous every 12 h. In those with severe illness, the first administration should be given as a 200 mg loading dose. Subsequent doses should then resume at the standard 100 mg every 12 h. In children weighing less than 45 kg, 1.1 mg/kg every 12 h should be given. The duration of therapy is discussed in sections pertaining to particular syndromes below.

3.2 Chloramphenicol

Chloramphenicol has long been considered the primary alternative to tetracyclines for SFGR, TGR, and scrub typhus. For the SFGR and TGR, the MICs are 0.25–2.0 μg/mL (Raoult and Drancourt 1991; Rolain et al. 1998). The MIC of chloramphenicol against *O. tsutsugamushi* is 1.25–2.5 μg/mL (Raoult and Drancourt 1991). Choramphenicol has poor in vitro activity against *E. chaffenesis* and *A. phagocytophilum* (Brouqui and Raoult 1992; Klein et al. 1997; Horowitz et al. 2001; Maurin et al. 2003; Branger et al. 2004). Although inexpensive and readily available in much of the world, severe adverse reactions such as aplastic anemia should temper its use when the risk to benefit ratio is not favorable. Chloramphenicol is no longer manufactured or available in its oral form in the U.S. In addition, there have been shortages of the intravenous formulation. Therefore, in the U.S., the drug is very difficult to obtain and may not be a viable treatment option when clinicians are faced with those with a severe allergery to drugs in the tetracycline class. Where available, and when drugs in the tetracycline class are absolutely contraindicated, chloramphenicol is given to adults at a dose of 500 mg every 6 h oral or intravenously. In children, the dose is 12.5 mg/kg every 6 h intravenously.

3.3 Fluoroquinolones

The fluoroquinolones are a class of relatively broad-spectrum antibiotics with in vitro activity to organisms in the genus *Rickettsia*. The MIC of readily available fluoroquinolones (i.e., ciprofloxacin, ofloxacin, and levofloxacin) range from 0.25 to 1.0 μg/mL to the SFGR and TGR tested (Jabarit-Aldighieri et al. 1992; Maurin and Raoult 1997; Rolain et al. 1998). Although ciprofloxacin has appeared effective against *O. tsutsugamushi* in vitro (Kelly et al. 1995) and in a mouse model (McClain et al. 1988), there have been clinical failures in humans treated with the antibiotics. Subsequent studies have detected mutations in the *gyrA* gene (target site for fluoroquinolones) of *O. tsutsugamushi* (Tantibhedhyangkul et al. 2010; Jang et al. 2013). The presence of this mutation in the organism's genome confers resistance and should preclude the use of fluoroquinolones in those with scrub typhus. Although *E. chaffeensis* is not

susceptible to fluoroquinolones, these agents have some activity against *A. phagocytophilum* (MIC for ofloxacin 1–2 μg/mL, ciprofloxacin 1–2 μg/mL, and levofloxacin 0.5–1 μg/mL) (Branger et al. 2004).

The fluoroquinolones have excellent oral bioavailability and are generally well-tolerated. Concomitant administration with divalent cations will inhibit the absorption of fluoroquinolones, so use of these medications in combination should be avoided. Fluoroquinolones are not recommended for routine use in children due to potential development of arthropathy as demonstrated in animal studies (Hooper and Strahilevitz 2015). Data regarding their use in SFGR and TGR are discussed in sections below. Ciprofloxacin is used at oral doses of 250–750 mg twice daily; ofloxacin is used at oral doses of 200–400 mg twice daily; and levofloxacin is used at oral doses of 250–750 mg once daily. Doses must be adjusted if there is renal impairment.

3.4 Macrolides/Ketolides

The macrolides consist of erythromycin, clarithromycin, and azithromycin. Josamycin, another macrolide, is available in other markets, but not in the U.S. These medications have in vitro activity against *Rickettsia* and *Orientia*. The oldest of these drugs, erythromycin, has MICs against SFGR and TGR of 4–8 μg/mL and 0.06–0.125 μg/mL, respectively (Raoult et al. 1988; Rolain et al. 1998). Clarithromycin and azithromycin are the newer macrolides available in the U.S. In regard to SFGR, MICs vary depending on the agent: *R. rickettsii* (2–8 μg/mL), *R. conorii* (1.0–16 μg/mL), and *R. akari* (0.25–2 μg/mL). The MIC of these drugs against TGR is 0.1–0.25 μg/mL (Maurin and Raoult 1993; Keysary et al. 1996; Ives et al. 1997). Macrolides are not active in vitro against *E. caffeensis* and *A. phagocytophilum* (Branger et al. 2004).

Both clarithromycin and azithromycin have excellent pharmacokinetic and pharmacodynamic properties, which result in high concentrations of the active drug in tissues and effector cells. They are safe in pregnancy. Although clarithromycin has many drug–drug interactions, azithromycin has much fewer. Use of either medication may be accompanied by gastrointestinal complaints (i.e., nausea, diarrhea, and abdominal pain), but they occur much less frequently than with erythromycin. The usual adult dose of clarithromycin is 500 mg twice daily. Azithromycin is typically started with a 500 mg loading dose on the first day, followed by 250 mg in subsequent days for indications such as community acquired pneumonia, but it has been studied using a variety of dosing schedules, including single dose regimens, depending on the infectious syndrome being treated (Sivapalasingam and Steigbigel 2015). When evidence is available, dosing schedules have been provided in the syndrome sections below.

Telithromycin is an erythromycin derivative classified in a separate antimicrobial class—the ketolides. Due to its enhanced pharmacokinetics, it is a once-daily medication. It has activity against several SFGR (*R. rickettsii*, *R. conorii*, *R. africae*) and

TGR with in vitro MICs of 0.5–1 μg/mL (Rolain et al. 2000). Although not studied in clinical illness caused by SFGR and TGR, it has demonstrated clinical effectiveness in those with scrub typhus. Unfortunately, reports of severe liver toxicity have prompted the FDA to relabel the medication with additional warnings. For this reason, macrolide therapy seems to be a safer and more reasonable alternative.

3.5 Rifamycins

Although rifamycins are effective in vitro against a variety of SFGR (*R. rickettsii*, *R. conorii*, *R. japonica*, *R. honei*, *R. sibirica*, *R. africae*, *R. parkeri*, and *R. slovaca*) and the TGR, with MICs of 0.06–1.0 μg/mL (Rolain et al. 1998), there is no clinical data to support their effectiveness in humans. In a small study of those with Mediterranean spotted fever, a 5-day course of rifampin was associated with several treatment failures and was inferior to a 1-day course of doxycycline (Bella et al. 1991). Rifamycins are also effective against *O. tsutsugamushi* in susceptability studies. There is clinical data to support its use in those with scrub typhus (see below) (Watt et al. 2000). Rifampin has MICs against *Ehrlichia chaffensis* and *Anaplamsa phagocytophilium* of 0.03 μg/mL–0.125 μg/mL and 0.03 μg/mL, respectively (Brouqui and Raoult 1992; Klein et al. 1997; Horowitz et al. 2001; Maurin et al. 2003; Branger et al. 2004). Rifampin, the most available rifamycin, can be dosed at 300 mg twice daily or 600 mg daily in adults. It has excellent oral bioavailability and is well-tolerated (Maslow and Portal-Celhay 2015).

4 Antibiotic Treatment of Clinical Rickettsioses

4.1 Rocky Mountain Spotted Fever

There are no prospective randomized clinical trials comparing various antibiotic regimens for the treatment of RMSF. The excellent in vitro susceptibilities and overwhelming retrospective clinical evidence support tetracyclines as the antibiotic class of choice (Walker and Blanton 2015). As mentioned above, the ease of dosing, tolerability, and bioavailability with food favor doxycycline as the drug of choice in this class. Although the drug has excellent absorption via the oral route, RMSF is often accompanied by gastrointestinal symptoms such as nausea and vomiting, which may necessitate hospitalization and parenteral administration. When a patient with RMSF is presenting with severe or moderate illness, a 200 mg oral or intravenous loading dose of doxycycline should be given prior to continuation of 100 mg every 12 h. When parenteral therapy is used initially, it can be switched to oral therapy as soon as the patient is able to reliably tolerate oral medications. It should be noted that oral tetracyclines, including doxycycline, can induce or worsen nausea. The usual course of treatment for those with RMSF is to continue for 3–5 days after the resolution of fever. This typically results in a 7-day course.

Chloramphenicol has long been considered an alternative treatment for RMSF, but it does not seem as effective as drugs in the tetracycline class. Analysis of clinical data from confirmed and probable cases of RMSF collected by the Centers for Disease Control demonstrated a higher case fatality rate when patients were treated with chloramphenicol compared to those treated with tetracyclines (7.6 % compared to 1.5 % with an odds ratio of 5.5) (Holman et al. 2001). Although available in other parts of the Americas, where RMSF is reported, in the U.S. chloramphenicol is either not available (oral formulation) or difficult to obtain (parenteral formulation). Although fluoroquinolones and macrolides have been used successfully with other spotted fever group rickettsioses, there are no available clinical studies to support or guide the use of other antimicrobial agents for RMSF. If faced with a history of severe hypersensitivity to doxycycline, and considering the potential severity of RMSF, doxycycline desensitization should be considered. Doxycycline desensitization protocols have been published, but their initiation requires close patient monitoring in the intensive care unit (Fernando and Hudson 2013; Stollings et al. 2014). Fortunately, hypersensitivity reactions to doxycycline are infrequent.

4.2 Other Spotted Fever Group Rickettsioses

There are many spotted fever group rickettsioses distributed throughout the world. *Rickettsia conorii*, the agent responsible for MSF, is the second most pathogenic SFGR after *R. rickettsii*. Although most of the following discussion involves studies in patients with MSF, these same principles can be intuitively applied to other SFG rickettsioses. As with infection from all the other pathogens discussed in this chapter, doxycycline is the antibiotic of choice. Unlike other SFGR, prospective studies have been performed in MSF comparing various antibiotic regimens. Short courses of doxycycline therapy in those with milder forms of MSF appear effective. Adults treated with a single day of doxycycline (200 mg dosed twice on the day of treatment) had similar outcomes to patients treated with 10 days of tetracycline hydrochloride (Bella-Cueto et al. 1987). In children with MSF, continuing doxycycline for 1 day after patients became afebrile had similar outcomes to those treated with a 7-day course (Yagupsky et al. 1987). Similar to RMSF, chloramphenicol has long been considered an effective alternative, but the risks of potential adverse events must be weighed against the potential benefits. Fortunately, alternatives exist for the treatment of infection with these other less virulent spotted fever group organisms.

In patients with mild to moderate MSF, fluoroquinolones have been shown to be effective. A 7-day course of ciprofloxacin (750 mg oral twice daily) was no different than doxycycline in regard to duration of fever, but there were fewer gastrointestinal complaints in the group that took ciprofloxacin (Ruiz Beltran and Herrero Herrero 1992b). None of the patients in this study had a severe form of MSF. Another description of five patients treated with ciprofloxacin reported success in all but one. The failure occurred in a man with acquired immunodeficiency syndrome who had

a very severe form of MSF (Raoult et al. 1986). Although fluoroquinolones looked to be an effective alternative in those with mild disease, there have been concerns regarding worse disease outcomes in those with MSF who have received fluoroquinolones (Botelho-Nevers et al. 2011). A proposed mechanism links the overexpression of a toxin-antitoxin system as demonstrated in cell culture experiments using *R. conorii* (Botelho-Nevers et al. 2012).

In those with milder forms of MSF, the newer macrolides may be an alternative in pregnant women, children, or those unable to take doxycycline. Although erythromycin has poor tolerability and has demonstrated disappointing results in those with MSF (Munoz-Espin et al. 1986), other macrolides such as clarithromycin and azithromycin appear effective for mild cases. In children, clarithromycin has been compared to chloramphenicol and has a shorter time to defervescence (Cascio et al. 2001). A 3-day course of azithromycin has been compared to a 5-day course of doxycycline in children and appears as effective (Meloni and Meloni 1996).

4.3 Typhus

The usual treatment of louse-borne epidemic typhus is doxycycline for 5–7 days. One caveat to consider is the setting in which the patient is being treated. Since typhus often occurs in large epidemics under conditions, which promote body louse infestations, short courses of mass treatment have been proposed. In these situations, a single 200 mg dose of oral doxycycline has been attempted (Perine et al. 1974; Raoult et al. 1998), but relapses have been documented (Huys et al. 1973). Therefore, such regimens should be used with great caution and should only be considered when resources are extremely limited. Chloramphenicol, where available, is an alternative therapy.

4.4 Murine Typhus

Tetracyclines are the treatment of choice for murine (endemic) typhus. Their successful use has been documented by a wealth of clinical experience that has been summarized elsewhere (Dumler 2012). Chloramphenicol is an alternative, but in a large retrospective analysis, it was associated with a longer time to defervesce when compared to doxycycline (4.0 days for chloramphenicol compared to 2.9 days for doxycycline). Murine typhus has also been successfully treated with fluoroquinolones, such as ciprofloxacin, but time to defervesce is even longer than the aforementioned antibiotics (4.2 days) (Gikas et al. 2004). It should be noted that treatment failures have also been reported with the use of fluoroquinolones (Laferl et al. 2002). The usual duration of treatment for murine typhus is 7 days.

4.5 Scrub Typhus

As with the organisms in the genus *Rickettsia*, there is overwhelming clinical experience supporting the use of tetracyclines for the treatment of infection with *Orientia tsutsugamushi*. Short courses of doxycycline have been used with some success. A single 200 mg dose of doxycycline was as effective as a 7-day course of tetracycline hydrochloride in one study (Brown et al. 1978). In another multicenter study performed in Korea, 3 days of doxycycline had similar outcomes to those treated with 7 days of tetracycline hydrochloride (Song et al. 1995). Because of reported relapses using abbreviated courses of doxycycline, duration of therapy as similar to those with RMSF should be used when possible. Chloramphenicol is an alternative treatment for scrub typhus, but it is associated with a longer febrile period and higher relapse rate as compared to patients treated with tetracyclines (Sheehy et al. 1973). In northern Thailand, where there are reports of patients who have had poor response to doxycycline (Watt et al. 1996, 1999), azithromycin has emerged as an alternative (Panpanich and Garner 2002; Fang et al. 2012). Both a single 500 mg dose of azithromycin and a 3-day dosing regimen have been shown to be as effective as a week course of doxycycline (Kim et al. 2004; Phimda et al. 2007). A 7-day course of rifampin has been studied in comparison to doxycycline with excellent results (Watt et al. 2000). As mentioned above, *O. tsutsugamushi* is intrinsically resistant to fluoroquinolones. They should not be used in scrub typhus (Tantibhedhyangkul et al. 2010; Jang et al. 2013).

4.6 Human Monocytotropic Ehrlichiosis

Tetracyclines are the treatment of choice for HME and infection with other *Ehrlichia* spp. Although no prospective clinical trials have been performed, case series show efficacy. With the use of tetracyclines, fever usually abates within 2 days of initiation (Fishbein et al. 1994). Early treatment with doxycycline has been associated with fewer complications and shorter hospital stays (Hamburg et al. 2008). Agents such as chloramphenicol, macrolides, and fluoroquinolones are not active and should not be used (Brouqui and Raoult 1992; Maurin et al. 2001; Branger et al. 2004). Although there is little published experience with rifampin in HME, its in vitro activity makes it an attractive alternative agent for HME during pregnancy.

4.7 Human Granulocytotropic Anaplasmosis

Treatment of HGA is similar to that of HME in that tetracyclines such as doxycycline are the treatment of choice. Rifampin is an alternative agent in pregnant women or children (see below). Chloramphenicol is not recommended.

Although fluoroquinolones appear to have some in vitro activity, they are not recommended as a treatment option for HGA, as there is a lack of robust clinical experience and a documented case of relapse in a patient taking levofloxacin (Wormser et al. 2006).

5 Considerations in Childhood and Pregnancy

Tetracyclines are known to cause staining of permanent teeth in developing children. Therefore, many fear the use of doxycycline in children with suspected rickettsioses. Studies evaluating tooth shade in children who have taken courses of doxycycline note no appreciable change in tooth color after short courses of the drug (Grossman et al. 1971; Todd et al. 2015). Considering the morbidity and mortality associated with *R. rickettsii* infection, the American Academy of Pediatrics endorses the use of doxycycline in children suspected of having RMSF ("Rocky Mountain spotted fever" 2015).

Tetracyclines have a number of effects that raise concern in the developing fetus and child. Deposition of the drug in the fetal skeleton may result in a temporary inhibition of bone growth (Cohlan et al. 1963), and the drug can also cause discoloration of deciduous teeth in children whose mothers received tetracyclines (Cohlan 1977). They have also been associated with maternal hepatotoxicity and pancreatitis during pregnancy (Herbert et al. 1982). Chloramphenicol, long considered the alternative for RMSF during pregnancy, has availability issues in the United States (see above). It is also associated with gray baby syndrome, which is characterized by abdominal distention, pallor, cyanosis, and vasomotor collapse. This is a concern, as transplacental concentrations of chloramphenicol can be as high as 50 % of that in maternal blood (Ross et al. 1950). These issues must be strongly considered when faced with a case of RMSF during pregnancy. When chloramphenicol cannot be obtained, doxycycline may be the only viable treatment option.

In children and pregnant women with less severe spotted fever group rickettsioses or murine typhus, when the risk for severe morbidity and mortality is much less than that of RMSF, azithromycin seems to be a reasonable alternative based on in vitro susceptibilities and limited patient experience. Fluoroquinolones are generally contraindicated in these groups. Scrub typhus can be treated with azithromycin (a regimen backed by clinical studies), but with the limited evidence available, pregnancy and neonatal outcomes are still poor (McGready et al. 2014). Although there is very little clinical experience with rifampin use for HME and HGA, in vitro susceptibility data intuitively suggest that it would be effective. There are a few published cases of children and pregnant women who were successfully treated for HGA with rifampin (Buitrago et al. 1998; Krause et al. 2003; Dhand et al. 2007).

6 Prevention

Measures to prevent the aforementioned diseases are generally aimed at avoiding contact with potential vectors. In respect to preventing spotted fever group rickettsioses, ehrlichiosis, and anaplasmosis, protective clothing such as long sleeves, pants, and high socks should be worn to protect individuals from the bite of ticks. Treatment of clothing with permethrin is effective at reducing the number of tick bites (Miller et al. 2011; Vaughn et al. 2014). When in tick-infested areas, people should perform frequent body checks for the presence of ticks. Attached ticks should be promptly removed with forceps with care to remove the imbedded mouthparts. In areas where the brown dog tick (*Rhipicephalus sanguineus*) is responsible for spotted fever group rickettsioses, treatment of yards and dogs with ascaricides is beneficial in curbing the tick population (Drexler et al. 2014) and therefore may be helpful for the prevention of disease transmission.

In the case of typhus group rickettsioses, hygiene and vector control play a major role in disease prevention. During times of poor, overcrowded, and unhygienic conditions (e.g., mass migration, war, natural disasters), body lice may proliferate and ignite an epidemic of typhus. The washing of blankets and garments in hot water will kill lice and their eggs. If this is not possible on a large scale, the mass treatment of clothed individuals with permethrin by use of a compressed air duster is endorsed by the World Health Organization (Darby et al. 1988; "Epidemic typhus risk in Rwandan refugee camps" 1994). Murine typhus was controlled in the United States after aggressive vector control programs using dichlorodiphenyltrichloroethane affected the rat-flea population enough to break the cycle of transmission to humans (Strandtmann and Eben 1953; Pratt 1958). Measures to control rat infestations have also been beneficial in the control of murine typhus (Traub et al. 1978).

Prophylaxis for scrub typhus can be given to those who may be deployed or traveling to an endemic area. A weekly 200 mg oral dose of doxycycline is effective, but the regimen must be strictly followed, as failure to adhere will result in a loss of efficacy (Olson et al. 1980; Twartz et al. 1982). Currently, there are no available commercial vaccines for the prevention of infection caused by any of the organisms belonging to the order *Rickettsiales*.

7 Summary

The key to treatment of infections caused by organisms in the order *Rickettsiales* is clinical recognition. When a rickettsiosis is suspected, prompt empiric antibiotic therapy should be initiated. Early treatment can quickly abate illness and prevent severe complications. Tetracyclines are the drug class of choice for all these infections, with doxycycline being the preferred agent. Where available, chloramphenicol

can be used for SFGR, TGR, and scrub typhus; the drug is not effective for ehrlichiosis and anaplasmosis. Other alternative agents are available, but their efficacy against the different genera of this order is not as generalizable as that of tetracyclines.

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Chapter 6
DHR-ICMR Guidelines for Diagnosis and Management of Rickettsial Diseases in India

Manju Rahi, M.D. Gupte, Anurag Bhargava, George M. Varghese, and Rashmi Arora

1 Introduction

Rickettsial infections are caused by a variety of obligate intracellular, Gram-negative bacteria of the genera *Rickettsia*, *Orientia*, *Ehrlichia*, *Neorickettsia*, and *Anaplasma*, belonging to the class Alphaproteobacteria. Bacteria of the genus *Rickettsia* are the well-known members of the order Rickettsiales. *Rickettsia* are divided into the typhus group and spotted fever group (SFG). *Orientia* spp. makes up the scrub typhus group (http://www.cdc.gov/). Rickettsial diseases are zoonoses where human beings are accidentally involved in a chain of transmission between trombiculid mites (chiggers), ticks or fleas, and animals (most commonly rodents).

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Classification of the Rickettsial diseases:

Diseases	Rickettsial agent	Insect vectors	Mammalian reservoirs
Typhus group			
(a) Epidemic typhus	*R. prowazekii*	Louse	Humans
(b) Murine typhus	*R. typhi*	Flea	Rodents
(c) Scrub typhus	*O. tsutsugamushi*	Mite	Rodents
Spotted fever group			
(a) Indian tick typhus	*R. conorii*	Tick	Rodents, dogs
(b) Rocky Mountain spotted fever	*R. rickettsii*	Tick	Rodents, dogs
(c) Rickettsial pox	*R. akari*	Mite	Mice

Among the major groups of rickettsioses diseases commonly reported in India are scrub typhus, murine flea-borne typhus, and Indian tick typhus.

2 Presenting Manifestations

Rickettsial infections are generally incapacitating and difficult to diagnose; untreated cases have case fatality rates as high as 30–45 % with multiple organ dysfunction, if not promptly diagnosed and appropriately treated (Batra 2007). The vast variability and non-specific presentation of this infection have often made it difficult to diagnose clinically. Given below are some of the presenting symptoms and signs of rickettsial infections:

Acute fever is the most common presenting symptom often associated with breathlessness, cough, nausea, vomiting, myalgia, and headache (Mathai et al. 2003; Vivekanandan et al. 2010).

An eschar at the site of chigger bite can be seen in early disease and is a useful diagnostic clue in scrub typhus, though its frequency varies from 7 to 97 % (Paris et al. 2013). Eschars are painless, ulcers upto 1 cm in size, with a black necrotic centre (resembling the mark of a cigarette burn) (Figs. 6.1 and 6.2). Usually a single eschar is found on the neck, axillae, chest, abdomen and groin, but multiple eschars have also been documented (Kaushik et al. 2014). Eschar on moist intertriginous surfaces (axilla, scrotum, perianal region) may be missed if not looked into carefully because they may lack the black scab and appear as shallow yellow-based ulcers without surrounding hyperemia.

Rash (in fair skinned people)

Though rash is considered a hallmark of rickettsial disease, it is neither seen at presentation nor in all patients. Presence of rash is common in spotted fever and extremely rare in scrub typhus. Rash usually becomes apparent after 3–5 days of the onset of symptoms. Initially, rash is in the form of pink, blanching, discrete maculae, which subsequently becomes maculopapular, petechial, or hemorrhagic (Rathi and Rathi 2010).

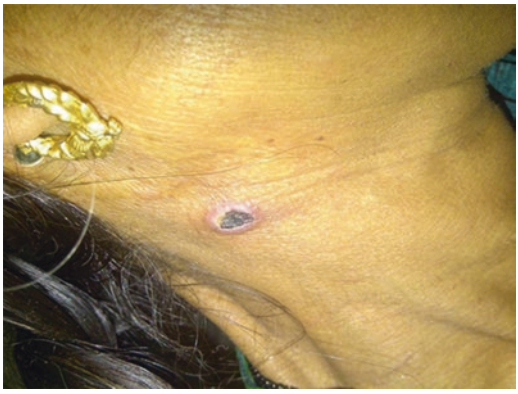

Fig. 6.1 Eschar at neck region

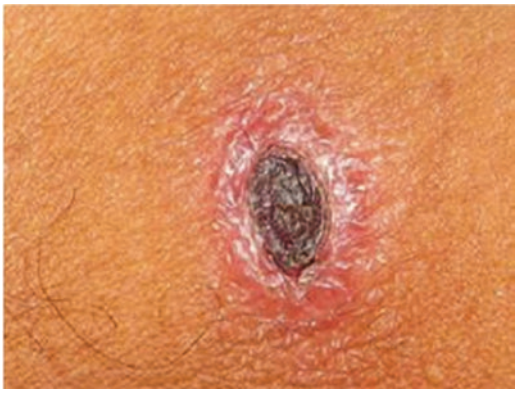

Fig. 6.2 Eschar with the typical central black scab with a cigarette burn like appearance on the skin

None of the clinical symptoms and signs including eschar are diagnostic of rickettsial disease. Therefore, epidemiological factors pertaining to geographical area, habitat, occupation, movement of the subject (vocational or recreational) could assist in reaching a diagnosis of rickettsial disease with certainty and initiating treatment on time.

The complications of scrub typhus usually develop after the first week of illness. Jaundice, renal failure, pneumonitis, acute respiratory distress syndrome (ARDS), septic shock, myocarditis, and meningoencephalitis are various complications known with this disease (Mahajan 2005).

Pneumonia is one of the most frequent complications of scrub typhus that manifests as a non-productive cough and breathlessness and leads to ARDS which could be life-threatening. Severe complications besides acute respiratory distress syndrome (ARDS) include hepatitis, renal failure, meningo-encephalitis and myocarditis; shock may occur in varying proportions of patients (Mahajan 2005).

Prompt antibiotic therapy, even based on suspicion, shortens the course of the disease, lowers the risk of complications, and in turn reduces morbidity and mortality due to rickettsial diseases. Currently, doxycycline is regarded as the drug of choice (Liu and Panpanich 2002).

There is a distinct need for physicians and health care workers at all levels of care in India to be aware of the clinical features, available diagnostic tests and their interpretation, and the therapy of these infections. Therefore, these guidelines are developed to help physicians towards correct diagnosis and treatment. Lack of awareness in physicians and community workers, as well as diagnostic delays, results in patients presenting to tertiary care centers with ARDS with severe complications resulting in a higher risk of mortality.

3 Guidelines for Management

3.1 Case Definition

3.1.1 Definition of Suspected/Clinical Case

Acute undifferentiated febrile illness of 5 days or more with or without eschar should be suspected as a case of Rickettsial infection. (If eschar is present, fever of less than 5 days duration should be considered as scrub typhus.) Other presenting features may be headache and rash (rash more often seen in fair persons), lymphadenopathy, multi-organ involvement like liver, lung, and kidney involvement. The differential diagnosis of dengue, malaria, pneumonia, leptospirosis, and typhoid should be kept in mind.

3.1.2 Definition of Probable Case

A suspected clinical case showing titres of 1:80 or above in OX2, OX19, and OXK antigens by Weil Felix test and an optical density (OD)>0.5 for IgM by ELISA are considered positive for typhus and spotted fever groups of *Rickettsiae*.

3.1.3 Definition of Confirmed Case

A Confirmed case is the one in which:

Rickettsial DNA is detected in eschar samples or whole blood by PCR.

Or

Rising antibody titers on acute and convalescent sera detected by indirect Immunofluorescence assay (IFA) or indirect immunoperoxidase assay (IPA).

3.2 Laboratory Criteria

There are various laboratory tests available for diagnosis of rickettsial diseases. Indirect immunoperoxidase assay (IPA) and immunofluorescence assay (IFA) are considered serological gold standards, but are available at laboratories with higher

level of facilities and expertise. Molecular diagnosis by PCR is available only at few centres in India. However, ELISA-based tests, particularly immunoglobulin M (IgM) capture assays, can be made available at secondary level and tertiary levels of health care like district hospitals and medical colleges.

Weil-Felix test, which is helpful in establishing presumptive diagnosis in diseases caused by members of typhus and spotted fever groups of *Rickettsiae,* can be considered at primary level of health care as they can be easily set up with moderate level of infrastructure and expertise at least in areas affected by scrub typhus.

3.2.1 Specific Investigations

Weil Felix: The sharing of the antigens between *Rickettsia* and *Proteus* is the basis of this heterophile antibody test. It demonstrates agglutinins to *Proteus vulgaris* strain OX19, OX2, and *Proteus mirabilis* OXK. Though this test lacks high sensitivity and specificity, it still serves as a useful and inexpensive diagnostic tool for laboratory diagnosis of rickettsial disease. This test should be carried out only after 5–7 days of onset of fever. Titer of 1:80 is to be considered possible infection. However, baseline titers need to be standardized for each region.

IgM and IgG ELISA: ELISA techniques, particularly immunoglobulin M (IgM) capture assays for serum, are probably the most of sensitive tests available for rickettsial diagnosis and the presence of IgM antibodies and indicate comparatively recent infection with rickettsial disease. In cases of infection with *O. tsutsugamushi*, a significant IgM antibody titer is observed at the end of the first week, whereas IgG antibodies appear at the end of second week. The cut off value is Optical Density of 0.5. Baseline titres need to be established keeping in view the regional variations.

Polymerase Chain Reaction (PCR): It is a rapid and specific test for diagnosis. It can be used to detect rickettsial DNA in whole blood and eschar samples. The PCR is targeted at the gene encoding the major 56 kDa and/or 47 kDa surface antigen gene. The results are best within first week for blood samples because of the presence of rickettsemia (*O. tsutsugamushi*, *R. rickettsii*, *R. typhi and R. prowazekii*) in the first 7–10 days.

Immunofluorescence Assay (IFA): This is a reference serological method for diagnosis of Rickettsial diseases and is considered the serological ‘gold standard’; however, cost and requirement of technical expertise limit its wide use. Therefore, it is recommended only for research and in areas where sero-prevalence of rickettsial diseases has been established and a reference facility is already available which has the necessary expertise required to conduct these tests.

Indirect Immunoperoxidase Assay (IPA): It gives comparable result as IFA, but requires special instrumentation and experienced personnel for interpretation of the test. Currently, we do not recommend any rapid test for diagnosis of scrub typhus as they need additional evaluation.

3.2.2 Supportive Laboratory Investigations

These are required as additional diagnostic clues and sometimes can indicate severity and development of complications. These investigations can assist in deciding upon appropriate management of patients.

(a) **Hematology**

- Total Leucocytes Count during early course of the disease may be normal, but later in the course of the disease, WBC count may become elevated to more than 11,000/cu.mm.
- Thrombocytopenia (i.e. <1,00,000/cu.mm) is seen in majority of patients.

(b) **Biochemistry**

- Raised Transaminase levels are commonly observed.

3.2.3 Imaging

- Chest X-Ray showing infiltrates, mostly bilateral (Figs. 6.3, 6.4 and 6.5).

3.3 Treatment

There is paucity of evidence based on randomized controlled trials for the management of rickettsial diseases including scrub typhus.

These guidelines for treatment cover the most common infection, the scrub typhus, murine typhus, and Indian Tick typhus.

Without waiting for laboratory confirmation of the Rickettsial infection, antibiotic therapy should be instituted when rickettsial disease is suspected.

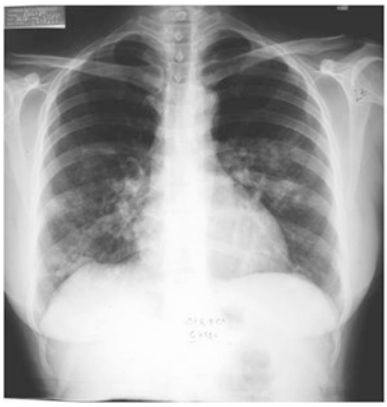

Fig. 6.3 X-ray of a patient with scrub typhus showing bilateral lower lobe interstitial infiltrates

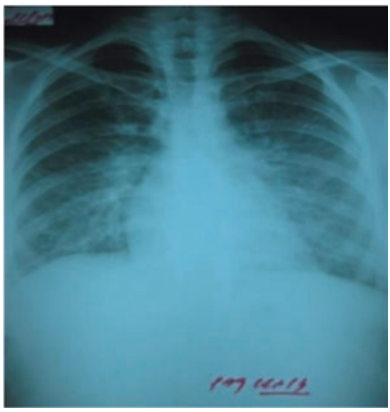

Fig. 6.4 X-Ray of a 30-year old woman presenting with fever for 10 days, non-productive cough for 5 days, and complaining of breathlessness. X-ray shows bilateral reticulonodular (interstitial) opacities in the lower lobes before treatment

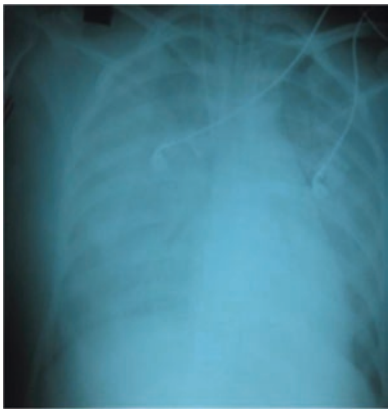

Fig. 6.5 X-ray of the same patient taken 2 days after admission to a tertiary care center with severe breathlessness. X-ray now shows bilateral extensive air-space consolidation suggestive of an acute respiratory distress syndrome

3.3.1 At Primary Level

The Healthcare provider needs to do the following:

(a) Recognition of disease severity. If the patients come with complications to primary health facility and the treating physician considers it as rickettsial infection, treatment with doxycycline should be initiated before referring the patient.
(b) Referral to secondary or tertiary centre in case of complications like ARDS, acute renal failure, meningo-encephalitis, multi-organ dysfunction. In addition to recommended management of community-acquired pneumonia, doxycycline is to be initiated when scrub typhus is considered likely.
(c) Patients with fever of more than 5 days where malaria, dengue, and typhoid have been ruled out; the following drugs should be administered when scrub typhus is considered likely—

Adults

(a) Doxycycline 200 mg/day in two divided doses for individuals above 45 kg for duration of 7 days. Patients should be advised to swallow capsules with plenty of fluid during meals while sitting or standing.
Or
(b) Azithromycin 500 mg in a single oral dose for 5 days.

Children

(a) Doxycycline in the dose of 4.5 mg/kg body weight/day in two divided doses for children below 45 kg.
Or
(b) Azithromycin in the single dose of 10 mg/kg body weight for 5 days.

Pregnant women

(a) Azithromycin 500 mg in a single dose for 5 days.
(b) Azithromycin is the drug of choice in pregnant women, as doxycycline is contraindicated.

3.3.2 At Secondary and Tertiary Care

(a) The treatment as specified above in uncomplicated cases.
(b) In complicated cases the following treatment is to be initiated—

- Intravenous doxycycline (wherever available) 100 mg twice daily in 100 mL normal saline to be administered as infusion over half an hour initially followed by oral therapy to complete 7–15 days of therapy.

- Or
  - Intravenous Azithromycin in the dose of 500 mg IV in 250 mL normal saline over 1 h once daily for 1–2 days followed by oral therapy to complete 5 days of therapy (Jang et al. 2014).
- Or
  - Intravenous chloramphenicol 50–100 mg/kg/day 6 hourly doses to be administered as infusion over 1 h initially followed by oral therapy to complete 7–15 days of therapy.
  - Management of the individual complications should be done as per the existing practices.

Doxycycline and/or Chloramphenicol-resistant strains have been observed in South-East Asia. These strains are sensitive to Azithromycin (Kim et al. 2007).

Disclaimer: These guidelines on diagnostic and treatment of rickettsial infections are based on a review of the currently available evidence and best practices and may be revised in light of future developments in the field.

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Part III
Anaplasma

Chapter 7
Anaplasma phagocytophilum in Sheep

Erik Georg Granquist

1 Introduction

Sheep are primarily wool, pelt, and food producing ruminants that are important in small- and large-scale settings. Sheep comprise myriads of breeds that are associated with a variety of cultures and husbandry traditions. Farmers and veterinarians endlessly toil to ensure appropriate biosecurity in the flock and to provide prophylactic treatments against diseases and disorders that may cause discomfort, disabilities, spread of disease, and production losses. Sheep farming and production are generally based on grassland systems and the rearing is semi-intensive or extensive in nature, depending on the outcome of production and geographical location. Since sheep are grazers, they move across a wide range of pastures and forest landscapes. Transhumance is a common husbandry practice in mountainous areas of various parts of Europe (Norway, Scotland) and the prealpine and alpine areas of Austria, France, Germany, Italy, Spain, and Switzerland (Eckert and Hertzberg 1994). Ticks are abundant in areas where the density of wild and domestic animals is high, thus sheep are prone to infections in their natural environments. The climate in Northern Europe varies from maritime, subarctic, and arctic to temperate. In Europe, lambing season coincides with the period in which ticks are reviving from dormancy in the spring, thus lambs may contract tick-borne diseases immediately after release on to pasture. In tropical and subtropical regions, sheep may be at risk of tick-borne infections all year around. Anaplasmataceae (proteobacteria family) includes the genera *Anaplasma*, *Ehrlichia*, *Neorickettsia*, and *Wolbachia*. Bacterial species belonging to this clade are known to colonize sheep, including *A. phagocytophilum* (Gordon et al. 1940), *A. ovis* (Kuttler 1984), *A. mesaeterum* (Uilenberg et al. 1979), *A. marginale* (Kuttler 1984), *Cowdria*

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ruminantium, *and A. platys* (Zobba et al. 2014). Common for these bacteria is the transmission by ticks, although the species of ticks vary. The focus of this chapter is on the microbial ecology of *A. phagocytophilum*, the infection and the disease in sheep. *A. phagocytophilum* is found in sheep, all across Europe and elsewhere. However, clinical disease is exclusively seen in Europe, and genetic variants of the bacterium show different degrees of virulence. No vaccines or optimal prophylaxis exist, and the treatment employs broad spectrum and long acting antibiotics. Easily available diagnostic tools and improved protective measures are more or less absent.

2 History

The agent of tick-borne fever (*Ehrlichia phagocytophila*) was together with *E. equi* and the agent of human granulocytic anaplasmosis, renamed as *A. phagocytophilum* in 2001 (Dumler et al. 2001). A condition named "skovsyge" is however described in Norwegian literature from 1780 and is consistent with tick-borne fever (Stuen et al. 1998b). Sheep was the first species to disclose *A. phagocytophilum* and the first formal discovery was made in Scotland in 1918 during a study on louping ill. Stockman described a condition on fever that lasted for 10 days in sheep, grazing on tick-infested pastures. The condition was transmissible by blood transfusion to other sheep and did not protect them from later Louping-ill infection (Stockman 1918). This disease was later described as an important tick-transmitted disease of sheep in Scotland. It was claimed that "tick-borne fever" was coexistent with louping ill, so that the investigation of this neurological disease in sheep was rendered difficult by the distinct type of infection. The unknown disease agent caused a thermal reaction in the sheep from the seventh day after inoculation with infected blood (MacLoed 1932). In September 1939, Dr. Gordon presented before the third international congress for microbiology (New York), the infective agent previously described as the agent of tick-borne fever. The agent was characterized as a rickettsia-like organism which could be observed in Giemsa stained blood smears, in the cytoplasm of the granular leucocytes, especially the neutrophil polymorphs (Gordon et al. 1940). A review of this evidence led Taylor et al. (1941) to suspect that tick infestation provided an unknown factor that was favorable to the development of a bacteremia, following superficial abscess formation in young lambs (Taylor et al. 1941). The suspicion was based on the observation that lambs infected with the tick-borne fever agent developed a neutropenia during the febrile phase. Dr. Foggie subsequently demonstrated the relationship between tick bite and the reduced resistance of young lambs to Staphylococci, injected intravenously during the neutropenic phase of tick-borne fever (Foggie 1947, 1948, 1957, 1959). The bacterium was later identified as a human infective agent in the United States (upper Midwest) (Bakken et al. 1994). This established the foundation for later research and clinical developments on *A. phagocytophilum*.

3 Etiology and Epidemiology

Tick-borne fever (TBF) in ruminants is also known as pasture fever and bovine- or ovine granulocytic anaplasmosis. The uncomplicated ailment is caused by the intracellular rickettsia called *A. phagocytophilum. A. phagocytophilum* is transmitted by hard ticks belonging to the *Ixodes persulcatus*-complex, and most commonly *I. ricinus* in Northern Europe (Daniel et al. 2015). Despite competent vectors have been identified elsewhere (Stuen et al. 2013a) and that serological as well as molecular evidence of infection in sheep exist across the globe (Gorman et al. 2012; Yang et al. 2015; Zhan et al. 2010), clinical tick-borne fever has never been reported outside Europe. The most vulnerable sheep are first time grazers and those purchased and introduced to tick-infested pastures for the first time (Stuen et al. 2013a). Approximately half of the >30 million sheep in the UK live on hilly and often tick-infested areas, and estimates show that nearly 300,000 lambs develop tick-borne fever each year, complicated by pyemia (Brodie et al. 1986). In Norway, approximately 500,000 lambs are exposed to ticks on pastures during each grazing season. Between 60 and 96 % of the lambs exposed, have been shown to carry *A. phagocytophilum* infection, resulting in an estimated 300,000–480,000 lambs possibly being infected annually (Stuen et al. 2002a; Grøva et al. 2011). Although the estimated indirect loss to *A. phagocytophilum* is high, the total costs may be much higher due to fatalities and crippling staphylococcal infections (Stuen et al. 2002a). Several studies have focused on the understanding of natural transmission cycles for *A. phagocytophilum*. The epidemiological cycles are poorly understood and involve different ecotypes that circulate in various host species and show differences in virulence (Dugat et al. 2015; Stuen et al. 2003a). Studies are based on extensive sampling of animals that are believed to be part of the three-host life cycle. Samples are studied by various genotyping approaches and alignments to create systematic views for linkage of bacterial variants to various hosts (Bown et al. 2007). This has to some extent, produced valuable information, but the complete complexity of genetic compositions with link to reservoir hosts is still far from surmounted. The *ankA* gene comparisons in sheep, dogs, humans, horses, cats, cows, bison, roe deer, and red deer isolates show that the sheep strains are much more diverse than human isolates. It also shows that sheep strains cluster together with human, dog, red deer, horse, and cat strains. However, isolates from roe deer belong to different clades (Scharf et al. 2011). This may support that sheep carry genetic variants, different from roe deer and that there are two separate transmission cycles existing for sheep and roe deer variants. Knowledge about genotypes in wild ruminants that have clinical relevance is preliminarily parse, however studies indicate that there is a shared flow of bacterial strains between red deer and sheep, but provides further support that roe deer belongs to a different clade of infection cycles than sheep and red deer (Stuen et al. 2013b). The highly discriminatory yet conserved *msp4* locus is being increasingly used for exploring the molecular epidemiology of *A. phagocytophilum* infections. A recent study reported 24 different *msp4* sequence types concurrently circulating in a Norwegian sheep flock (Ladbury et al. 2008). In contrast to the *ankA*

gene, typing based on the *msp4* gene showed a clustering of the red deer isolates distinct from sheep (Stuen et al. 2013a). Experimental infection trials in which sheep were inoculated with both sheep and red deer isolates gave ambiguous results, showing receptiveness in sheep for deer isolates. The sheep had milder disease sequelae with the deer variant than the with the sheep variant, evidenced by higher bacteremia and a stronger serological response for the sheep isolate (Stuen et al. 2010). This emphasizes that even though deer and sheep isolates may share phylogenetic clusters and other similarities in genetic structures, the difference in clinical outcomes may be due to far more complex and unknown mechanisms than hitherto revealed. The heterogeneity of isolates from domestic and wild ruminants may be the result of genetic exchange via recombination between *A. phagocytophilum* strains in multiply infected, highly tick-exposed animals (Huhn et al. 2014). Regarding the 16S gene, one study found six different genotypes associated with clinical disease in sheep from Norway (Granquist et al. 2010a). The importance of gaining knowledge about transmission cycles is high with regard to prevention of disease in livestock. Professed beliefs may have misled farmers and caretakers of sheep to believe that roe deer are the ones to blame for tick-borne diseases in humans and livestock. The picture may be more nuanced than it appears when looking into the transmission dynamics of different variants among ruminants. In red deer, it has been shown that serum complement factors may dilute the bacterial load of *Borrelia* spp. in ticks (Kurtenbach et al. 2002; Bhide et al. 2005). This has however not been shown for *A. phagocytophilum*. Since sheep are extensive grazers compared to cattle, cattle strains may be more homologous than sheep strains. Sheep are also exposed to seasonal transhumance across a variety of territories, thus increasing their likelihood of exposure to different ticks, and thus diverse *A. phagocytophilum* strains (Dugat et al. 2014). Molecular studies have shown that transmission of *A. phagocytophilum* between cells of different species depends on host specific elements at the receptor level. This may explain why certain strains of *A. phagocytophilum*, e.g., the Norwegian var1 strain (Gen. Bank M73220), are so far refractory to culture in commercially available cell lines, like the human HL-60 cell line (Carlyon et al. 2003; Herron et al. 2005; Reneer et al. 2006). All age groups of lambs have been shown to be of epidemiological importance for the maintenance of *A. phagocytophilum* in *I. ricinus* populations (Stuen and Bergström 2001a, b).

4 Infection and Transmission

Besides tick bites, livestock can be infected by blood transfusion and through vaccination if the same needle is used repeatedly on several individuals (Reinbold et al. 2010). An experimental infection in ewes resulted in one ewe giving birth to an infected lamb, 5 weeks after inoculation, suggesting that transplacental infection with *A. phagocytophilum* is possible (Reppert et al. 2013). However, no reports about transplacental infections are available from studies in the field. *A. phagocytophilum* shows a partiality for phagocytic cells and especially neutrophil

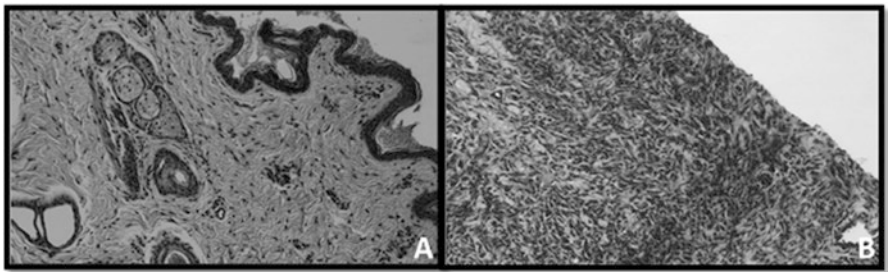

Fig. 7.1 (**a**) Sheep skin, control biopsy outside the tick bite, normal dermis. (**b**) Sheep skin, biopsy from a tick bite. Dermatitis accompanied by moderate edema. Massive leukocyte infiltration

granulocytes (de la Fuente et al. 2015). During tick feeding, neutrophil-associated inflammatory responses are modulated by various stimuli deployed by the tick sialome (Beaufays et al. 2008; Guo et al. 2009; Heinze et al. 2012). Histologic examination of the sheep skin reveals massive infiltrations of polymorphonuclear cells at the tick bite site (Granquist et al. 2010a) (Fig. 7.1). The bacterium also modulates the distribution of potential host cells and infected neutrophils by inducing cytokine secretion and their receptors (Akkoyunlu et al. 2001; Scorpio et al. 2004), and promoting the loss of P-selectin and L-selectin (Choi et al. 2003). The bacterium further interacts with host cell ligands (Granick et al. 2008; Park et al. 2003) by surface-exposed proteins known as adhesins (Ojogun et al. 2012; Yago et al. 2003) in order to facilitate internalization in the host cell (Wang et al. 2006). The orchestration of vector and bacterial interactions with the defensive mechanisms of the host animal, thus seems to promote the infection.

Because of the short-lived nature of circulating neutrophils, the role of these cells in establishing and maintaining infection has been questioned (Herron et al. 2005), however to date little is known about alternative cellular components involved in the invasion and colonization of *A. phagocytophilum* in sheep (Granick et al. 2008). Interestingly *A. phagocytophilum* delays the apoptosis in neutrophils, ensuring the extended life span of these cells and bacterial survival (Scaife et al. 2003). *A. phagocytophilum* persists in sheep between seasons of tick activity, thus being available for transmission at times when the vector is active (Granquist et al. 2010c; Thomas et al. 2012). In Northern and Central Europe, clinical tick-borne fever is usually diagnosed from the beginning of April until the middle of November. However, most cases are seen between the middle of May and the middle of June (Estrada-Peña et al. 2004; Lindgren and Gustafson 2001). This is the period in which ticks are most active (Stuen et al. 2002b). The activity of *I. ricinus* varies greatly between geographic regions and climatic zones and ticks usually revive from diapause when the temperatures surpass 5–8 °C (41–46 °F) and the activity is lower when temperatures are high (Lindgren and Gustafson 2001). The prevalence of *A. phagocytophilum* in *I. ricinus* can vary extensively between seasons and geographical locations which may also influence the risk of transmission to sheep (Grzeszczuk and Stańczak

2006). Bacteremia is expressed as cyclic peaks where each cycle represents the emergence of one or multiple clones of the bacterium. These clones express unique immune-dominant outer membrane proteins, known as major surface protein MSP-2 (Granquist et al. 2008). The low level of circulating organisms, detected between periods of bacteremia (Granquist et al. 2010c), may indicate temporary clearance of infected cells or possible margination of infected granulocytes to endothelial surfaces. The driver for creating generations of antigenically different organisms is most likely the specific immune response seen in sheep, that is evident subsequently to the arise of new immune-dominant antigens which results in a persistent infection (Granquist et al. 2008, 2010b, c).

5 Clinical Signs and Pathogenesis

The infection can cause extensive lamb losses on tick pasture (Øverås et al. 1985). Tick-borne fever is more common in young lambs, than gimmers and adult sheep, and maternal antibodies seem to give no protection against tick-borne fever (Stuen et al. 1992). The age-related resistance is probably due to acquired immunity that develops after exposure. Despite this, studies have shown that superinfections may occur by genetically distinct organisms of *A. phagocytophilum* (Ladbury et al. 2008; Stuen et al. 2009). Although *A. phagocytophilum* is the second most common tick-borne infection in humans in the United States, no naturally infected clinical cases have been reported in sheep from the US. One experimental infection with the Webster strain (GenBank U02521.7) and the MRK strain (GenBank No. AY530196) resulted in only subclinical to mild infections in sheep (Gorman et al. 2012). Additionally, one experimental study from Oklahoma, US, discovered that sheep could become infected by the NY18 strain, but these sheep did not show clinical signs of infection (Reppert et al. 2014). Another trial infected sheep with an equine strain of *A. phagocytophilum*, which resulted in a subclinical, but hematologically visible infection. Then, upon challenge with a sheep strain, the sheep became clinically ill (Stuen et al. 1998a). The horse strain did not produce any protection against further strains. The severity of the disease is influenced by several factors, such as questing activity of the ticks; variants of *A. phagocytophilum* present in the tick population; feeding time of the tick; prevalence of other tick-transmitted pathogens; and host factors such as age, immune status, and body condition of the animal (Stuen et al. 2012). The infectious dose does not seem to be important for the clinical outcome of disease (Stuen and Artursson 2000) and it is believed that only one or a few organisms is enough to establish the infection. After tick bite, the incubation period is usually between 3 and 14 days and the infection can be subclinical, mild, or severe. The infection itself is seldom fatal, but it may deteriorate by secondary infections (Stuen et al. 2013a). The most pronounced clinical signs during the acute phase are dullness and severe pyrexia which often tangents 42 °C (108 °F). However, the fever reaction may vary according to the age of the animal, the variant of *A. phagocytophilum* involved, and the immunologic status of the host animal

(Stuen and Longbottom 2011). During the peak period of bacteremia, up to 90 % of the granulocytes may be infected (Woldehiwet 2010). A pronounced neutropenia ($<0.7 \times 10^9$ neutrophils/Liter) is observed following the onset of fever, which may last for up to 2 weeks (Stuen et al. 1998c). The fever is not typically coherent with the number of neutrophils infected (Stuen et al. 1998c). The bacterium has the ability to extend the life span of the normally short-lived neutrophils by inhibition of apoptosis. By staying inside vacuoles of neutrophils, *A. phagocytophilum* eludes the hostile intracellular environment of the host cell and creates a safe haven for further propagation in the host (Gokce et al. 1999a; Scaife et al. 2003). As neutrophils and their chemical armaments constitute the primary hurdle against invading bacteria and fungi, lambs are liable to suffer from secondary infections while immune suppressed (Stuen et al. 2003b). The secondary infections are typically bacterial in nature and caused by invading *Staphylococcus aureus* (Foggie 1956), *Chlamydia psittaci* (Munro et al. 1982), *Listeria monocytogenes* (Grønstøl and Ulvund 1977), or *Pasturella* spp. (Brodie et al. 1986; Øverås et al. 1993). A *Staphylococcus* infection may typically lead to a condition called tick pyemia. These infections result in crippling, paralysis, arthritis, septicemia, neurological affection, and pneumonia (Woldehiwet 2006). After, or accompanying *A. phagocytophilum* infection, lambs may be infected by viruses whose disease sequelae may be catalyzed by the immune suppression. Typical viruses may be parainfluenza type 3 virus (Batungbacal and Scott 1982), louping ill virus (Reid et al. 1986), and orf virus (Gokce and Woldehiwet 1999b). Early studies showed that ticks are unlikely to carry *S. aureus* by acting as true vectors for the bacterium; however, ticks may introduce the bacterium from skin surfaces or natural orifices through the tick bite site to the internal blood vessels by acting as a mechanical vector. The established *S. aureus* infection is then favored by the reduced immunity of the lamb, as result of *A. phagocytophilum* infection (Foggie 1947). During the neutropenic state, the number of infected cells is reduced. This reduction can be reversed by injecting sheep with corticosteroids (Woldehiwet and Scott 1982). It has been questioned whether the reduction in infected neutrophils is due to margination of infected cells to endothelial surfaces or migration to the spleen, however no evidence has shown this in sheep yet. This is important in order to understand how the bacterium persists in the host animal (Granquist et al. 2010a). Dexamethasone treatment has led to diminished clinical signs in horses, which points to the fact that clinical signs and suffering associated with tick-borne fever are dependent on pro-inflammatory responses (Davies et al. 2011). Infected sheep also show a marked decrease in the number of B (LCAp220+) lymphocytes, (γδ T-cells, CD4+, CD5+, and CD8+(CD4−CD8−) T-cells during early infection. This reduction in T-helper and T-suppressor cells returns to normal levels after 13–16 days of infection (Whist et al. 2003; Woldehiwet 1991). Later in the infection, CD8+ have been observed to increase, possibly reflecting an immune response (Whist et al. 2003). Besides secondary infections, complications may also include abortion (García-Pérez et al. 2003; Giudice et al. 2011), when ewes are moved on to tick-infested pastures during late gestation (Woldehiwet 2006). When rams are infected in late autumn, they may appear with reduced fertility when the breeding season commence due to impaired spermatogenesis (Woldehiwet and Scott 1993).

Infected lambs may show poor development and reduced weight gain, even with mild clinical symptoms (Stuen et al. 2002a). Sheep usually recover from the clinical disease after about 14 days; however, they usually remain persistently infected for several months. Very young lambs (1–2 weeks old) may react with less clinical symptoms than older lambs (Stuen et al. 1992).

6 Diagnosis

The first clinical signs of tick-borne fever are usually, recognized by sheep caretakers. However, since the disease is associated with discomfort and production loss, veterinary attention is required in most cases. Knowledge about the occurrence of ticks and *A. phagocytophilum* in vicinity of the flock or farm premises is important as anamnestic information for the field diagnostic approach. The infection may often not manifest itself in the animal before complications by secondary infections make them display discomfort or that they succumb to systemic disease. Depending on the type of husbandry, monitoring rectal temperature and visual appearance of the flock may aid the timing of implementing prophylactic actions at an early stage of an outbreak to avoid the most severe clinical symptoms and secondary infections. Although a clinical diagnosis can be made by field practitioners, a laboratory confirmation is usually required to verify the diagnosis (Woldehiwet 2010). The conservative diagnostic method is to collect anticoagulated blood from the jugular vein. The sample is carried to the laboratory for preparation of thin blood smears which can be stained by May-Grühnwald Giemsa (Woldehiwet 2006), Wright stain, or LeukoStat stain (Dumler et al. 2005). Polymorphonuclear cells usually settle on the feathered edge and in the monolayer of the smear. The proportion of infected cells can be determined by examining 400 neutrophil granulocytes from each smear (Stuen et al. 2002c). Light microscopy of blood smears taken in the initial fever period is normally sufficient to reveal the diagnosis. Stained with May-Grünwald Giemsa, the organisms will appear as bluish cytoplasmic inclusions (morulae) in monocytes and granular leucocytes, especially neutrophils (Foggie 1951). Electron microscopy may also confirm the diagnosis of acute *A. phagocytophilum* infection in blood or organs. Single or multiple organisms are then identified in clearly defined cytoplasmic vacuoles (Tuomi and von Bonsdorff 1966). Hematology will reveal a marked neutropenia. Immunohistochemistry on tissue samples can be performed to visualize organisms in peripheral tissues (Granquist et al. 2010b; Lepidi et al. 2000) (Fig. 7.2).

Several molecular techniques are used to identify DNA or RNA from *A. phagocytophilum* in blood and tissue samples in sheep. Polymerase chain reaction, reverse line blot hybridization, and *16S* rDNA gene sequencing were some of the early applications in the identification of *A. phagocytophilum* and its genetic variants (Alekseev et al. 2001; Christova et al. 2003; Stuen et al. 2002b). For diagnostic purposes multiplex PCRs can be used to detect and differentiate simultaneous infections in ticks, animals, and humans (Chan et al. 2013). Specific primers for

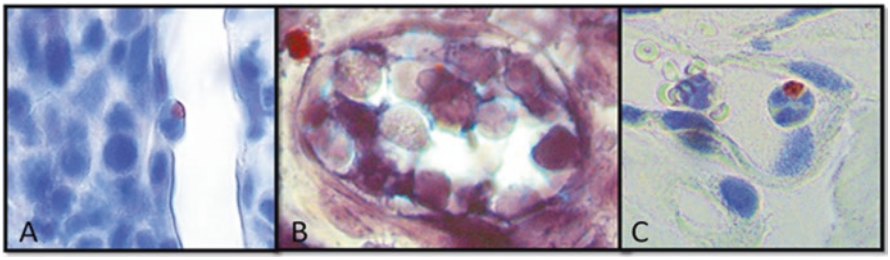

Fig. 7.2 Sheep skin biopsy of tick bite. (**a**) *A. phagocytophilum* morula inside marginating leukocyte (MSP2) immunostain, original magnification (×1000). (**b**) *Anaplasma phagocytophilum* seen at the rim of a blood vessel. (**c**) *Anaplasma phagocytophilum* morula inside a polymorphonuclear cell. Monoclonal antibodies for these micrographs were kindly provided by Dr. Stephen J. Dumler

amplification of genetic elements in *A. phagocytophilum* are available, such as *16S* rDNA, *groEL, msp4, Ank*, and the *p44/msp2* genes (Kang et al. 2011; Silaghi et al. 2011). Both conventional and real-time PCR are used for diagnostic purposes in sheep. The real-time approach can also be used to quantify genes or the bacteremia (genome equivalents) by comparing C_t values to a standard curve, usually made by amplifying a plasmid containing the desired gene fragment in a known quantity (Granquist et al. 2010c). PCR techniques should be combined with at least one of various nucleotide sequencing techniques to confirm the specificity of the amplified product. Alternatively, PCR products can be analyzed directly or indirectly after cloning, by using restriction endonuclease mapping or hybridization (Granquist et al. 2008). The presence of specific antibodies may support the clinical diagnosis. However, serum conversion and the antibody production have been shown to depend on the genetic variant of *A. phagocytophilum* involved in the infection (Stuen et al. 2003a). Paired sera taken 14–21 days apart may differentiate acute from persistent infection, but serum antibodies tend to diminish just a few weeks after infection (Stuen et al. 2003a; Granquist et al. 2010b). Complement fixation test, countercurrent immune electrophoresis, and indirect immunofluorescent antibody (IFA) assay are in use to detect antibodies against *A. phagocytophilum* in sheep sera (Paxton and Scott 1989; Stuen et al. 2003a; Webster and Mitchell 1988). Since IFA titers may persist for some weeks after the primary infection, the test may not be suitable for determination of the acute or persistent phase of infection (Paxton and Scott 1989). Lambs have been shown to appear seronegative 4–6 weeks after inoculation with *A. phagocytophilum* and still being able to transmit the infection by blood transfusion to naïve lambs. The results suggest that serology is not a good indicator for assessing recovery from persistent infection (Stuen and Bergström 2001a, b). Several ELISA tests have also been developed and tested in sheep (Alleman et al. 2006; Granquist et al. 2010b; Woldehiwet and Yavari 2012). It would be beneficial from both clinical and research perspectives to have more efficient serological assays available from diagnostic laboratories, facilitating herd

level testing and population surveys. A SNAP®4Dx® ELISA and other rapid tests are commercially available for in-house identification of *A. phagocytophilum* antibodies in dog sera, but the SNAP®4Dx® has also successfully been used on sheep sera in the first weeks of infection (Granquist et al. 2010b). Succumbed sheep should generally undergo necropsy to disclose any warnings of emerging diseases or herd outbreaks. A typical carcass appearance with tick-borne fever is the enlarged spleen, which is up to four times the normal size, accompanied by subcapsular bleedings (Øverås et al. 1993; Stuen et al. 2013a). Previously, the enlarged spleen was regarded pathognomonic for tick-borne fever in sheep (Lepidi et al. 2000; Stuen and Olsson Engvall 1999). Microscopically, the spleen has been observed with slight diminution of lymphoid cells accompanied by increased numbers of macrophages and neutrophils in the sinuses (Lepidi et al. 2000). In addition, paracortical hyperplasia may be present in the lymph nodes with occasional hemophagocytic cells and the liver may show mild periportal lymphocytic infiltration and small aggregates of macrophages with adjacent apoptotic hepatocytes (Lepidi et al. 2000). Other typical pathological changes have not been described, except for secondary pneumonia, signs of septicemia, arthritis, and disseminated abscessation.

7 Treatment

The most effective curative treatment against *A. phagocytophilum* in sheep is the intramuscular injection of oxytetracycline either daily or as depot injection (Woldehiwet and Scott 1993). The recommended dosage varies between countries and readers are remitted to national regulatory authorities for guidance on trade and use of antibiotics. Tetracycline is also effective as prophylaxis, when given at the time of, or 5 days prior to infection (Brodie et al. 1986, 1988). The oxytetracycline treatment may reduce numbers of circulating and infected neutrophils and markedly reduce clinical signs (fever) in sheep. After treatment for 5 days, the blood is however still infective, meaning that clearance of infection with oxytetracycline is ineffective (Stuen and Bergström 2001a, b). Treatment should always follow a thorough clinical evaluation of the single patient or sheep flock. The veterinarian should always consider providing analgesic treatment and anti-inflammatory drugs. Due to depression and fever, sheep may be reluctant to seek feed and water, thus fluid therapy and nutritional support may be considered. The danger of rapidly emerging antimicrobial resistance in target and nontarget bacteria must be taken into consideration with any use and choice of antibiotics. For prophylaxis against *A. phagocytophilum*, the principle is to remove or reduce the tick infestation on the animal (Stuen et al. 2012). This is mostly achieved by the application of a variety of chemical acaricides that can be administered to sheep by parenteral injections, per os treatment (ruminal bolus or drenches), plunge dips, jetting, application of ear tags, neck collars, or by pour-on preparations (Wall 2007). Available chemicals used in the treatment of ectoparasites act either systemically, following uptake of the compound from tissues, or by direct contact with the target tick following external

application, all resulting in repellent and acaricidal effect on the tick. Virtually all ectoparasiticides are neurotoxins, exerting their effect on the tick nervous system (Taylor 2001). The strategic tick control implies the application of acaricides during the periods of tick burdens. For disease control measures, this involves treating the flock throughout the entire grazing season. Thus, the treatment with acaricides is becoming relatively expensive, time consuming, and must usually be repeated several times during the grazing season due to the short action of these chemicals. Studies have shown that ticks may infest animals already 13–14 days after application of acaricides, and they became infected by *A. phagocytophilum* while treated (Henderson and Stevens 1987; Mitchell et al. 1986; Stuen et al. 2012). Acaricidal drugs should be safe to handlers, safe toward nontarget organisms, and have rapid environmental decay (Dekeyser 2005) as they enter into the environment through disposal of waste material, excretion of feces and urine by grazing animals, and through spillage during application or disposal of the compound (Beynon 2012). As an example, arsenic, which is a carcinogen to humans, was frequently used earlier in plunge dips for both sheep and cattle, which has resulted in the contamination of soil and groundwater (Sarkar et al. 2004). Most treatments with ectoparasiticides cause chemical residues in the milk, meat, and wool and are subjects to withdrawal periods. The success of treatment depends on several factors such as correct dosage, frequency of application, method of application, climatic factors, bioavailability of the drug, the resistance status in ticks, and the tick burden. This type of treatment will kill ticks: either in the environment or while feeding on the animal. Available compounds belong to the organophosphates, carbamates, formamidines, organochlorines, pyrethrins, and pyrethroids groups. The supply of these drugs will vary between countries. In most areas of the world, especially one-host ticks have been reported to become resistant to a number of acaricides (Abbas et al. 2014). Resistance is far less prevailing in multihost ticks like the *I. ricinus*. The loss of certain effective acaricides to resistance and the potential for tick populations to develop resistance to those remaining may contribute to an upsurge in tick-transmitted diseases (Taylor 2012). Vaccines, in general, are often more feasible and cost effective and more environmentally friendly than chemical control methods. There are currently no commercial vaccines available against *A. phagocytophilum* in any species. Infection followed by treatment has shown the establishment of immunity against reinfection (Brodie et al. 1988), suggesting that a possible way of obtaining immunity is by inoculation with virulent strains or by early turnout on tick-infested pastures before treating lambs with long acting oxytetracyclines. This method has, however, several ethical drawbacks and practical disadvantages as well as offering limited protection against superinfections in lambs (Stuen et al. 2009). An effective vaccine against tick-borne fever has been demanded by sheep farmers and veterinary practitioners for years. The lack of such vaccines is partly due to the difficulties in obtaining high quality genome data from livestock-associated strains of the pathogen and detailed information on the genetic structure of different strains. Current work is ongoing to produce effective vaccine candidates against *A. phagocytophilum* for sheep by methods involving reverse vaccinology approaches, genetic manipulation techniques, and attenuation procedures. Biological control of ticks is becoming a

parallel and attractive approach to manage ticks. However, biological control of ticks has been difficult because ticks have few natural enemies. Studies so far have concentrated on insectivorous birds, symbiotic bacteria, parasitoid wasps, *Bacillus thuringiensis*, entomopathogenic fungi (*Metarhizium anisopliae* and *Beauvaria bassiana*), and nematodes (Fernandes et al. 2012; Granquist et al. 2014; Samish et al. 2004). The main challenge remains to create a sustainable biological control of ticks in the natural habitat that does not cause disturbances in the ecosystem. Rickettsia that may be of particular interest in biological control of ticks are *Wolbachia pipientis* and *Midichloria mitochondrii* (Granquist et al. 2014).

8 Other *Anaplasma* Species in Sheep

Anaplasma ovis is an intraerythrocytic rickettsial pathogen that is widespread in sheep across the world. The bacterium is transmitted by many species of ticks and has been found in Europe (Hornok et al. 2007; Torina et al. 2010), Asia (Yang et al. 2015), Africa (Ndung'u et al. 1995), and the USA (Splitter et al. 1956; Goff et al. 1993). Usually the infection in domestic sheep is subclinical, but if immunosuppression coincides with the infection, a severe anemia may accrue during the acute phase (Hornok et al. 2007). In this period the animal may display fever, dullness, weight loss, abortion, lowered milk production, and paleness or jaundice on mucous membranes (Rymaszewska and Grenda 2008). The bacterium has been shown to persist in animals that recover from the acute infection (Palmer et al. 1998). Unlike *A. centrale*, *A. ovis* does not protect cattle against *A. marginale* infection (Kuttler 1984). In addition, *A. ovis* has not been observed infecting or establishing persistent infection in cattle, which is beneficial in terms of cograzing with infected sheep (Kocan et al. 2010). The infection is diagnosed most easily by blood smears stained with Giemsa and serological tests either using *A. marginale*-derived antigens or by using *A. ovis* antigens (Sumption 2000). In addition, molecular assays have been developed (de la Fuente et al. 2007). Other *Anaplasma* spp. may establish subclinical infections in sheep like *A. bovis* (Ben Said et al. 2015), *A. marginale* (Kuttler 1984), and *A. mesaeterum* (Uilenberg et al. 1979). Oxytetracycline is effective in severe clinical cases.

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Part IV
Ehrlichia

Chapter 8
Exit Strategies of *Ehrlichia*

Sunil Thomas

1 Introduction

The entry and exit of intracellular pathogens from host cells are crucial steps in the infectious cycle; however, the mechanism is not clearly understood in many intracellular pathogens. It has recently emerged that microbial exit is a process that can be directed by organisms from within the cell, and is not simply a consequence of the physical or metabolic burden that is imposed on the host cell (Hybiske and Stephens 2007, 2008).

Ehrlichia are obligately intracellular bacterium that thrives in the monocytes and macrophages and are transmitted to vertebrates through tick bites. Human monocytotropic ehrlichiosis (HME) was first reported in 1987 (Maeda et al. 1987). Since then, development of murine models of persistent and lethal ehrlichiosis has greatly facilitated understanding of the pathogenesis and mechanisms of host defenses against ehrlichial infections. In general, microorganisms can disseminate after host cell lysis via necrotic or apoptotic cell death, or by spreading from cell to cell (Hagedorn et al. 2009). Until recently, the mechanism by which *Ehrlichia* are released from host cells or how they gain entry into cells was not demonstrated (Li and Winslow 2003; Maender and Tyring 2004; Rikihisa 2010a, b).

Recently, in a mouse model of monocytotropic ehrlichiosis, we demonstrated by eastern blotting that the heat shock protein 60 (Hsp60/GroEL) is highly post-translationally modified in the non-virulent *E. muris*, compared to the highly virulent strain IOE (*Ixodes ovatus* ehrlichia) (Thomas et al. 2009). Based on this observation we generated an anti-*Ehrlichia*-specific Hsp60 antibody and used it to observe *E. chaffeensis*, *E. muris*, or IOE in cell culture. This chapter explains the exit strategies of the intracellular bacterium *Ehrlichia*.

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2 Morphology of *Ehrlichia*

Before delving to the exit strategies of *Ehrlichia*, it is imperative to know the morphology of the pathogen. *Ehrlichia* are Gram-negative bacteria, usually round or ovoid, non-motile bacteria that reside and grow in cytoplasmic vacuoles derived from an early endosome, forming loose to condensed aggregates of bacteria termed morula (morula = mulberry-like structure) in monocytes and macrophages (Paddock and Childs 2003) (Fig. 8.1). The bacteria stain dark blue to purple with Romanovsky-type stains (Fig. 8.2). Though most of the strains of *Ehrlichia* are observed in monocytes and macrophages, *Ehrlichia ewingii* infects neutrophils. Infection with *E. ewingii* may delay neutrophil apoptosis (Xiong et al. 2008). The key to the successful survival inside the host cell is by preventing fusion of the phagosome and lysosome (Barnewall et al. 1997). Another factor that contributes to the successful intracellular survival of *Ehrlichia* is inhibition of host cell apoptosis. *Ehrlichia* morulae also interact with mitochondria and inhibit mitochondrial metabolism (Liu et al. 2011).

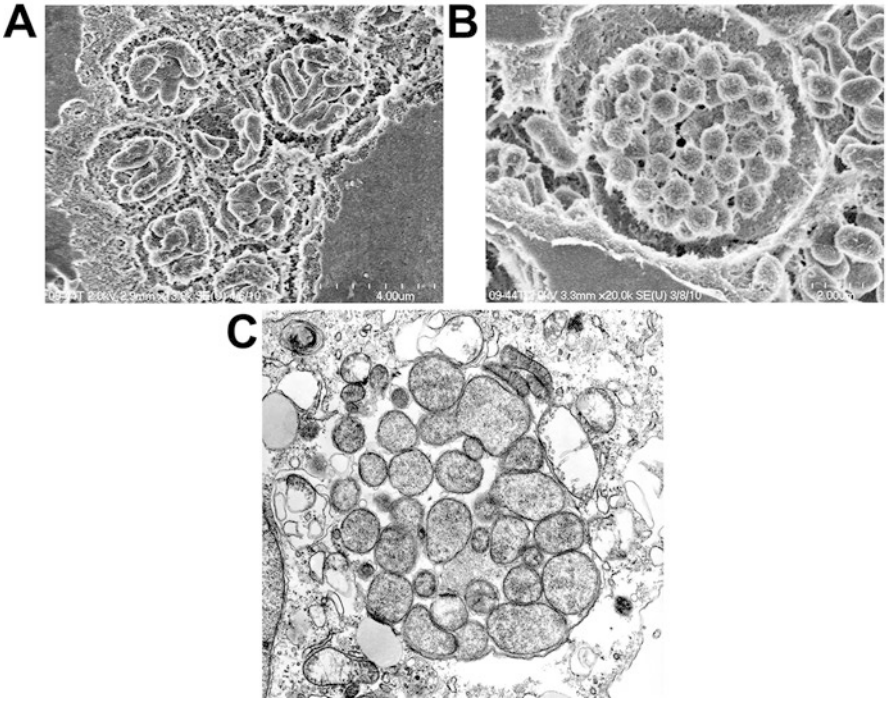

Fig. 8.1 Morula of *Ehrlichia muris*. Scanning electron micrograph of *E. muris* morula in DH82 host cells (**a**) reticulate cells and (**b**) dense cored cells. (**c**) Transmission electron micrograph of *E. muris* morula (reticulate cells) (Original image: magnification X32,000)

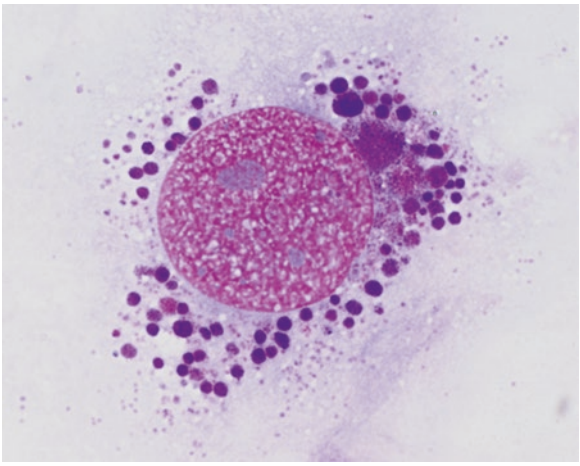

Fig. 8.2 *E. muris*-infected DH82 cell stained with Diff-Quik stain. The purple-colored *E. muris* morulae surround the host nucleus

Two distinct morphologic forms of *Ehrlichia* are identified in host cells, reticulate and dense-cored cells. The reticulate cells measure 0.7–1.9 μm, are of pleomorphic morphology with DNA and ribosomes distributed throughout the bacterial cytoplasm (Fig. 8.3). Whereas, the infectious dense-cored cells are predominantly coccoid bacteria and measure 0.4–0.6 μm in diameter, characterized by concentration of ribosomes and chromatin, and these predominate at early and late time points of infection (Fig. 8.4). Both cell types replicate by binary fission, and both demonstrate a Gram-negative-type cell wall, characterized by a smooth-contoured cytoplasmic membrane and a generally ruffled outer membrane, separated by a periplasmic space. The infectious dense-cored form of *Ehrlichia* transforms into reticulate forms 2–4 days post-infection, and later they undergo binary fission for about 2 days. Intermediate forms are seen 4–5 days post-infection and large numbers of dense-cored cells are observed 5–6 days after infection. The host cells rupture after 6–7 days of infection. Morulae range from 1.0 to 6.0 μm in width and contain 1 to >40 organisms of uniform or mixed cell types (Paddock and Childs 2003; Zhang et al. 2007). The intramorular space may contain a fine, striated fibrillar matrix that may be fibrillar ehrlichial antigen apparently shed from the surface of the cell wall (Fig. 8.5) (Popov et al. 1995; Paddock et al. 1997). The cellular morphology of *Ehrlichia* at several stages of its growth is shown in Fig. 8.6. In cell culture and infected human cells, host cell mitochondria are frequently apposed to the margins of morulae (Paddock and Childs 2003). Unlike other Gram-negative bacteria, *E. chaffeensis* lacks the genes for the biosynthesis of lipopolysaccharide (LPS) and most genes for the biosynthesis of peptidoglycan; thus, it does not produce LPS or peptidoglycan (Lin and Rikihisa 2003).

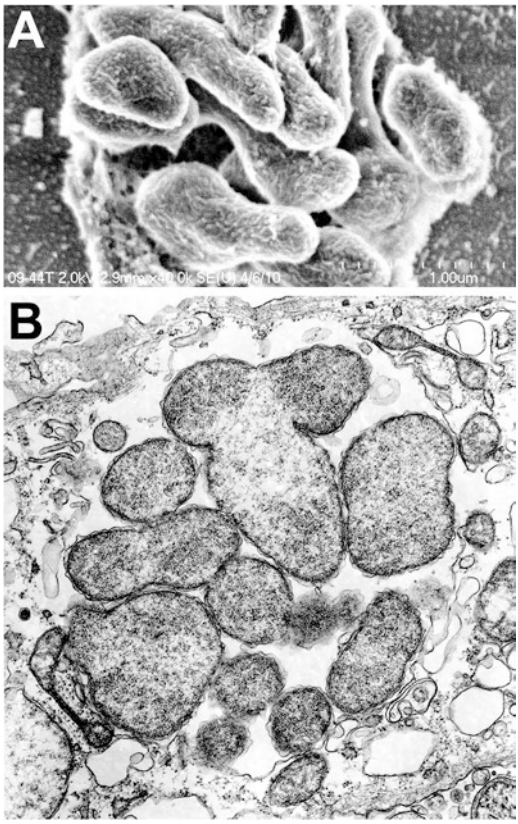

Fig. 8.3 Reticulate cells of *Ehrlichia muris*. (**a**) Scanning electron micrograph of *E. muris* reticulate cells and (**b**) Transmission electron micrograph of *E. muris* reticulate cells (Original image: magnification X47,000)

3 *Ehrlichia* Induce Filopodia in Infected DH82 Cells

The intracellular pathogens *E. chaffeensis* and *E. muris* are maintained in vitro in the DH82 monocyte cell line. Usually *Ehrlichia* are observed under a microscope after infection of uninfected DH82 cells with the bacterial pathogen. The 60 % confluent uninfected DH82 cells are seeded with *Ehrlichia*-infected DH82 cells and incubated for a further 3–4 days, when the DH82 cells achieve high confluence (no void between host cells), and all of them infected with *Ehrlichia. Ehrlichia* was observed after seeding around 1000 infected DH82 cells per slide so that they were separated from one another after 16 h of culture. After 16 h culture, the *E. muris*- and *E. chaffeensis*-infected DH82 cells were probed with the *Ehrlichia* Hsp60 antibody. By 16 h filopodia were observed in 30 % of DH82 cells infected with *Ehrlichia* (3 % in uninfected DH82 cells; $p < 0.0001$). Filopodia were extended from the polar ends of spindle-shaped *Ehrlichia*-infected host cells (*E. chaffeensis*: Fig. 8.7a; *E. muris*: Fig. 8.8a–d; uninfected DH82 cell: Figs. 8.7d and 8.8e; *E. muris*-infected

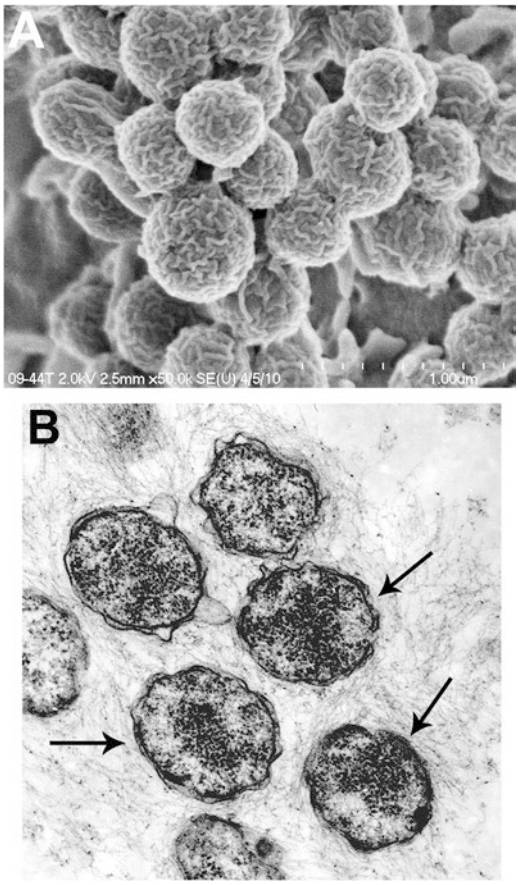

Fig. 8.4 Dense cored cells of *Ehrlichia*. (**a**) Scanning electron micrograph of *E. muris* dense cored cells and (**b**) Transmission electron micrograph of IOE dense cored cells (Original image: magnification X47,000)

DH82 cell without primary antibody: Fig. 8.7f) or from the non-polar sides of the cells when they contained many bacteria (*E. chaffeensis*: Fig. 8.7b). Filopodia of infected cells extended to the neighboring host cell (*E. chaffeensis*: Fig. 8.7b, *E. muris*: Fig. 8.8d). If host cells were not present in the vicinity of an infected cell, the leading edge of the filopodia of *Ehrlichia*-infected cells formed a flattened fan-shaped structure where the *Ehrlichia* cell cluster were contained (Fig. 8.7c). The bacterial cell cluster containing fan-shaped structure further developed its own filopodium (not shown). It is not known how the bacterial cell cluster-filled fan-shaped structure disintegrates and the contents released. Nevertheless, we have observed cluster of bacterial cells in vitro lying on host cells which we speculate are released on disintegration of the fan-shaped structure at the end of filopodium (Fig. 8.9).

Macrophage filopodia contain a meshwork of actin filaments and surround foreign organisms during phagocytosis (Hartwig et al. 1977); and cytochalasin D is known to inhibit actin polymerization (Rosania and Swanson 1996). Phalloidin has

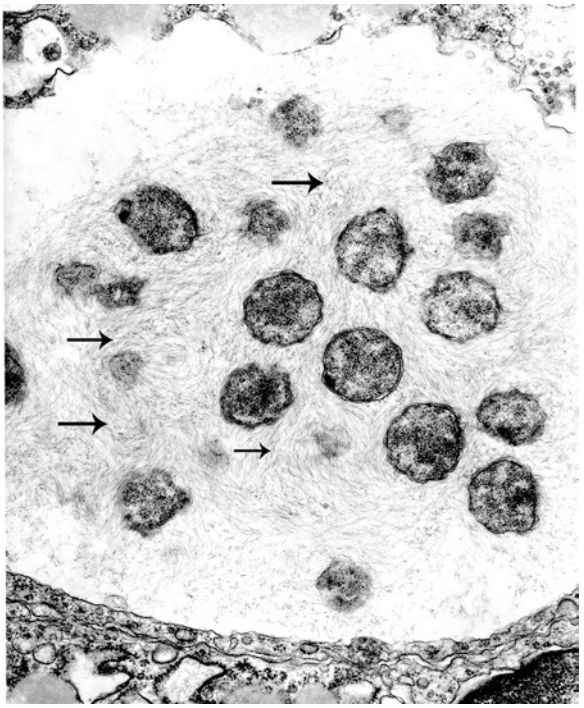

Fig. 8.5 Fine striated fibrilles are visualized in the morular matrix of IOE (*Arrows*) (Original image: magnification X47,000)

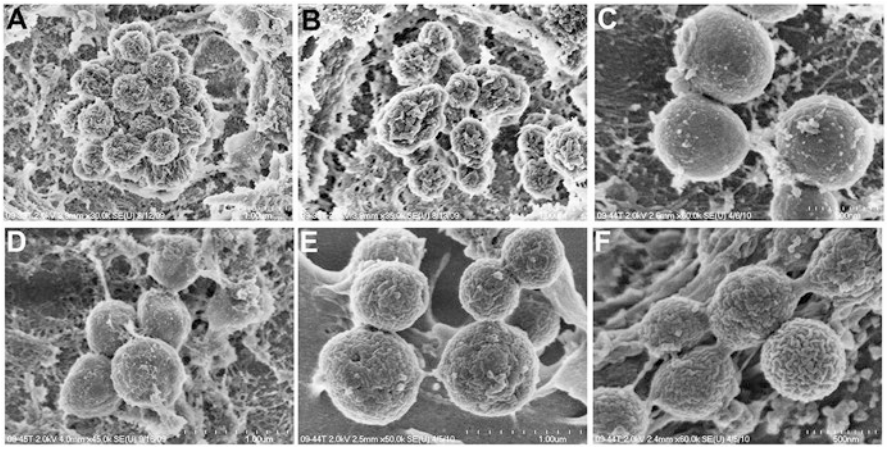

Fig. 8.6 Scanning electron micrographic collage of the morphology of *Ehrlichia*. (**a**–**c**) *E. chaffeensis*. (**d**–**f**) *E. muris*. Note the cell wall of the bacteria during various stages of division

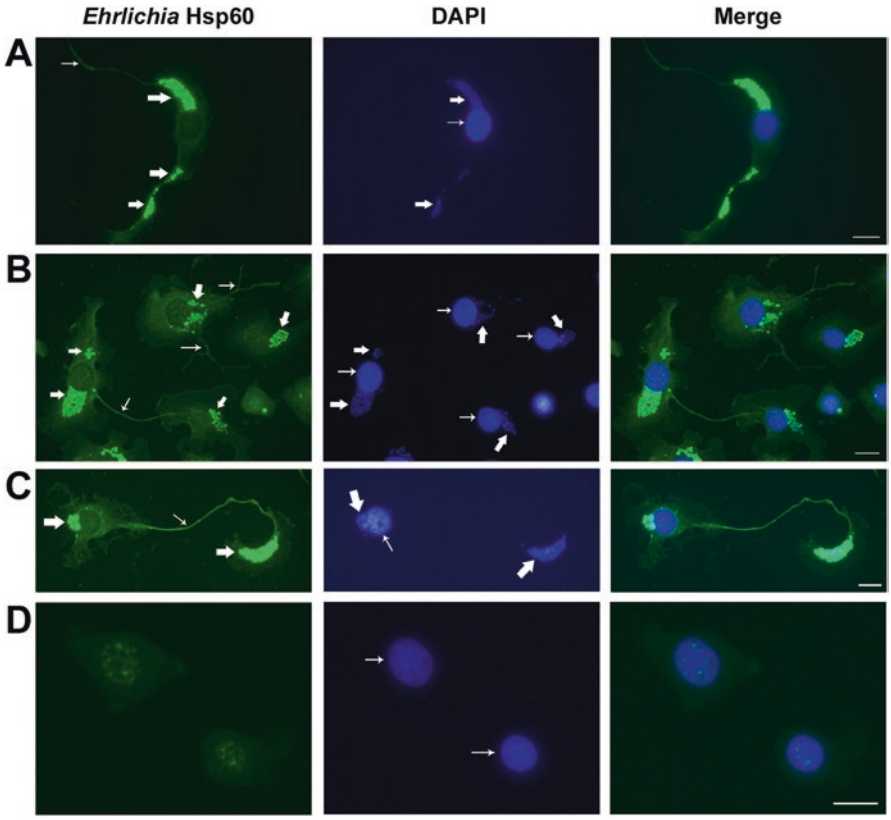

Fig. 8.7 *Ehrlichia* are contained in the filopodia of DH82 cells. (**a**) Filopodia extended from the polar ends of the *E. chaffeensis*-infected DH82 cell. *Left*: *E. chaffeensis*-infected DH82 cell probed with anti-Hsp60 antibody. *Thick arrow* indicates *E. chaffeensis* intracellular colonies and *thin arrow* indicates filopodium. *Middle*: *E. chaffeensis*-infected cell stained with DAPI. *Thick arrow* indicates morulae of *E. chaffeensis* stained with DAPI and *thin arrow* indicates host nucleus. *Right*: Merged figure. Scale bar, 25 μm. (**b**) Filopodia of *E. chaffeensis*-infected DH82 cells extended to neighboring cells. (**c**) When host cells were not in the immediate vicinity, the leading edge of an *E. chaffeensis*-infected DH82 cell formed a flattened fan-shaped structure filled with the pathogen. (**d**) Uninfected DH82 cell

a high affinity for actin, and phalloidin conjugated to Alexa 594 are used to detect actin in the filopodia. Filopodia stained with phalloidin-Alexa 594 were intensely red, whereas DAPI stained the host nucleus as well as the DNA of *E. chaffeensis* (Fig. 8.10a–d, uninfected DH82 cell: Fig. 8.10e). Filopodia formation was observed within an hour after culturing the infected DH82 cells. By 24 h the average length of a filopodium in infected cells was 120 μm (Fig. 8.10f). Filopodia measuring more than 10 times longer than the diameter of the host cell are sometimes observed during *Ehrlichia* infection. The filopodium is normally used by the macrophages or monocytes to probe for microorganisms and engulf them. However, the infectious

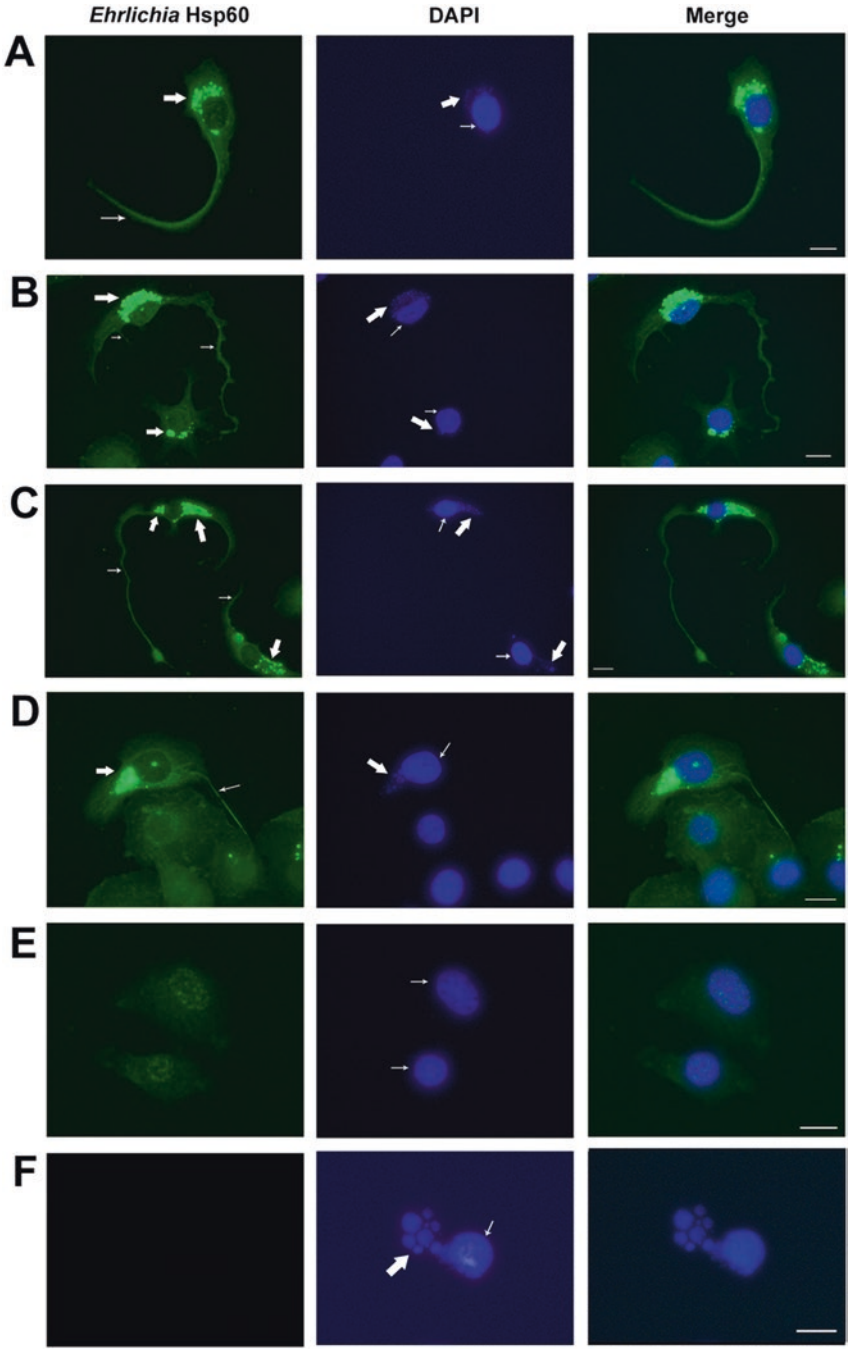

Fig. 8.8 *E. muris* is associated with the filopodia of DH82 cells. (**a**–**c**) Filopodium extending from the cell body of an *E. muris*-infected DH82 cell. *Left*: *E. muris*-infected DH82 cell probed with anti-*Ehrlichia* Hsp60 antibody. *Thick arrow* indicates *E. muris*, and *thin arrow* indicates filopodium.

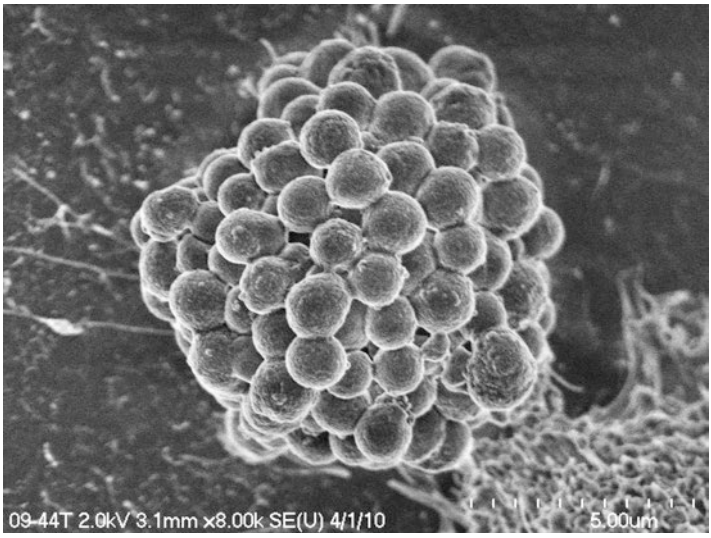

Fig. 8.9 SEM of a cluster of *E. chaffeensis* cells observed in vitro on DH82 cells. The fan-shaped structure at the end of the filopodium may disintegrate thereby releasing the cluster of bacterial cells

pathogens may use the filopodia to enter into host cells. The ultimate goal of any organism is to replicate and disseminate its progeny; microorganisms including bacteria are no exception. *Ehrlichia* bacteria use the filpodia as a vehicle to attach to neighboring cells. The major advantage of using the filopodia is non-recognition of the pathogens by the host immune system, thus evading death.

It is not possible to view the inside contents of a cell by scanning electron microscope (SEM). Hence, Scotch tape is used to mechanically break open the host cells and to observe the *Ehrlichia* inside DH82 cells by SEM. SEM of the mechanically opened cells demonstrated the presence of *Ehrlichia* in the filopodia of the DH82 host cells (Fig. 8.11a–f). On contact with a new host cell, the pathogens from the fan-shaped flattened structure (observed at the tip of the filopodium) were in a location where they can pass to the neighboring cell (Fig. 8.11e, f). Further, observation of cell membranes deformed from within by intracellular ehrlichiae revealed the opportunity for bacterial intrusion into the adjacent cells (Fig. 8.11g–i). These observations suggested that *Ehrlichia* passed from one host cell to another without entering the extracellular space.

Fig. 8.8 (continued) *Middle*: *E. muris*-infected cell stained with DAPI. *Thick arrow* indicates DNA of *E. muris* stained with DAPI, and *thin arrow* indicates host nucleus. *Right*: Merged figure. Scale bar, 25 μm. (**d**) Filopodium of an *E. muris*-infected DH82 cell extended to a neighboring cell. (**e**) Uninfected DH82 cells. (**f**) Absence of *Ehrlichia* Hsp60 primary antibody resulted in absence of staining *E. muris* in infected DH82 cells, but DAPI stained the *E. muris* DNA and DH82 nucleus

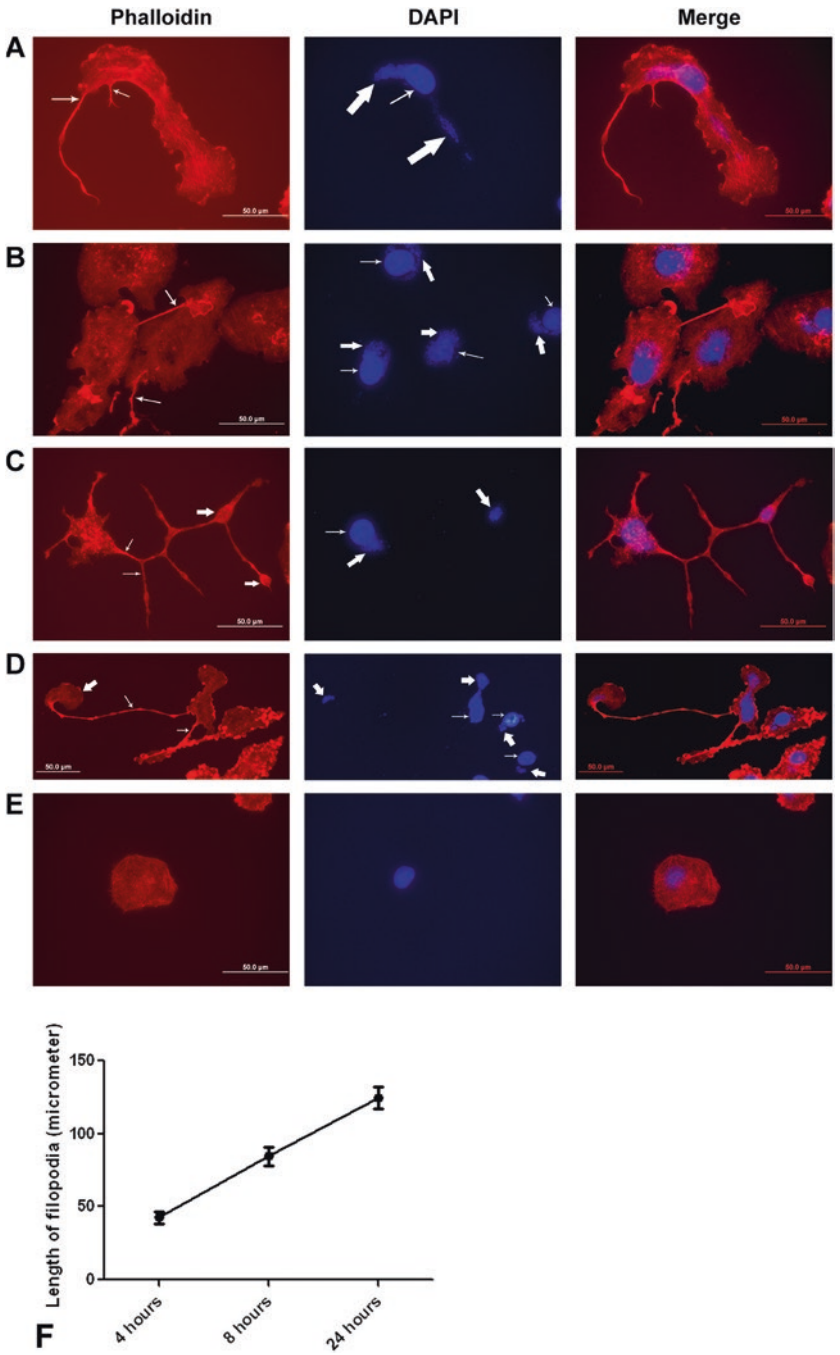

Fig. 8.10 Actin is a major protein of filopodia induced during *Ehrlichia chaffeensis* infection. *Left*: *E. chaffeensis*-infected DH82 cell probed with phalloidin. *Thin arrows* indicate filopodia. *Middle*: *E. chaffeensis*-infected cell stained with DAPI. *Thick arrow* indicates morulae of *E. chaffeensis* stained with DAPI, and *thin arrow* indicates host nucleus. *Right*: Merged figure. Scale bar, 50 μm.

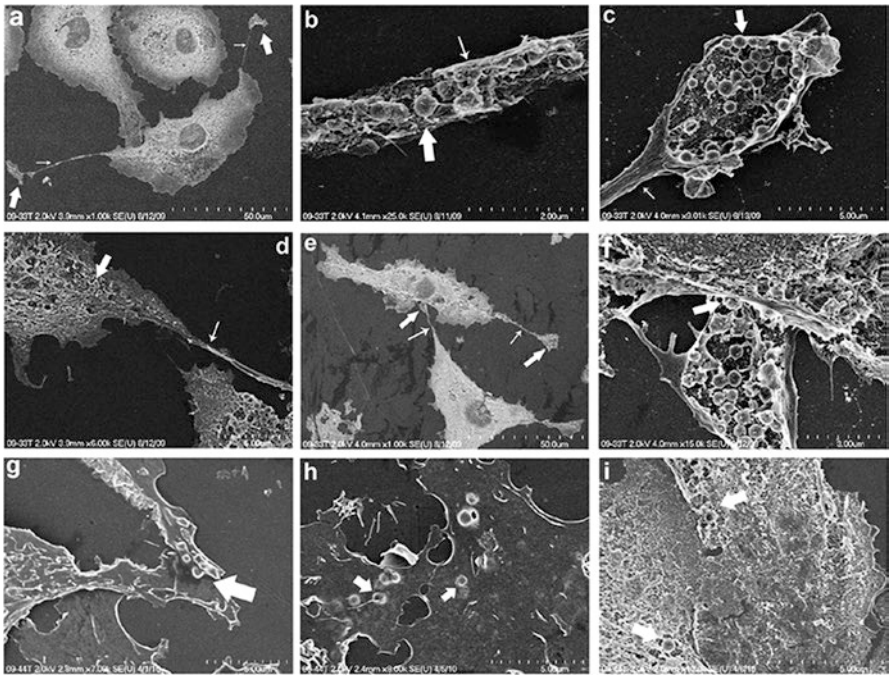

Fig. 8.11 *Ehrlichia* is transported through the filopodia of the host cells as observed under a scanning electron microscope. Scanning electron micrographs of DH82 cells infected with *E. chaffeensis*. (**a**) *E. chaffeensis* are observed in the filopodia of DH82 cells. The *thick arrow* indicates the flattened fan-shaped structure at the leading edge of the filopodium (indicated by *thin arrows*). (**b**) *Ehrlichia* bacteria in a filopodium from which the cell membrane has been removed. The *thick arrow* indicates an *Ehrlichia*. (**c**) A flattened fan-shaped structure filled with *Ehrlichia* from which the cell membrane had been removed. The *thick arrow* indicates *Ehrlichia*, and the *thin arrow* indicates a filopodium. (**d**) A filopodium that extended from an *Ehrlichia*-infected DH82 cell. (**e**) Low magnification of an *Ehrlichia*-infected host cell filopodium in contact with a neighboring cell. The *thick arrows* indicate the flattened fan-shaped structures, and the *thin arrows* indicate the filopodia. (**f**) High magnification of a flattened fan-shaped structure from which the cell membrane has been removed at the leading edge of an *Ehrlichia*-infected cell (depicted in figure **e**) in contact with the neighboring host cell. The *thick arrow* indicates an *Ehrlichia*. (**g**) Intracellular *Ehrlichia* deforming the overlying cell membrane at the junction of a neighboring cell. (**h**) Localization of *Ehrlichia* (*thick arrow*) deforming the overlying cell membrane of adjacent cells. (**i**) *Ehrlichia* seen in adjacent cells of a cracked open DH82 host cell

Fig. 8.10 (continued) (**a**) Filopodia extended from an *E. chaffeensis*-infected DH82 cell. (**b**) Filopodium of *E. chaffeensis*-infected DH82 cell extended to a neighboring cell. (**c**, **d**) *Ehrlichia* are contained in a long filopodium that had a flattened fan-shaped structure with no host cells in the immediate vicinity. *Thick arrow* indicates the flattened fan-shaped structure at the leading edge of the filopodium. (**e**) Uninfected DH82 cell. (**f**) Lengths of filopodia of DH82 cells infected with *E. chaffeensis* ($n = 25$)

4 Inhibition of Actin Polymerization in Host Cells Infected with *Ehrlichia* Prevents Filopodia Formation and Localizes the Pathogen in the Periphery

Actin and microtubules are involved in the formation of filopodia; hence, we determined the effect of the actin inhibitor, cytochalasin D on the transport of the pathogen in *Ehrlichia*-infected monocytes. Cytochalasin D inhibited filopodium formation in both *E. chaffeensis*- (Fig. 8.12a) and *E. muris*-infected cells (Fig. 8.12c, d, f, g). Ehrlichiae were confined to the periphery of the macrophages. *Ehrlichia* are transported through the filopodia so as to avoid the host immune system while the pathogen passed from cell to cell. The actin inhibitors latrunculin B, wiskostatin, and blebbistatin inhibited filopodium formation; whereas treatment of *E. muris*-infected DH82 cells with nocodazole (microtubule inhibitor) did not inhibit filopodium formation (Thomas et al. 2010).

To confirm indeed that filopodium is required for intercellular transport of *Ehrlichia*, the *E. muris*-infected DH82 cells were labeled with carboxyfluorescein succinimidyl ester (CFSE) followed by seeding with non-labeled uninfected DH82. Alternately, the uninfected DH82 cells can also be labeled with CFSE and seeded with DH82 cells infected with *E. muris*. The filopodia/pseudopodia from the infected DH82 cells were in close proximity to the neighboring uninfected DH82 cells and *Ehrlichia* was also observed in the uninfected DH82 cells after 16 h of culture (Fig. 8.13a–c). In the presence of cytochalasin D there was absence of any filopodia in the *Ehrlichia*-infected DH82 cells, and there was also no infection in the neighboring uninfected cells (Fig. 8.13d–e).

The amount of *Ehrlichia* increases if it is transferred intracellularly through the filopodium where it could infect neighboring uninfected cells, thereby multiplying in these cells. Whereas, inhibition of filopodia formation could decrease the pathogen and these could be quantitated by quantitative real-time polymerase chain reaction (RT-PCR). The uninfected DH82 cells were seeded with *E. muris*-infected DH82 cells (in the presence and absence of cytochalasin D) and after 24 h the bacterial load was quantitated by RT-PCR. When the uninfected DH82 cells were seeded with *E. muris*-infected DH82 cells in the presence of cytochalasin D, the bacterial load decreased, whereas in the absence of cytochalasin D the bacterial load increased (Fig. 8.13g). The data confirmed that induction of filopodia formation in host cells by *Ehrlichia* is indeed an exit strategy of *Ehrlichia*.

Fig. 8.12 (continued) (**e**) Uninfected DH82 cells treated with cytochalasin D and probed with *Ehrlichia* Hsp60 antibody. (**f**) Scanning electron micrograph of *E. muris*-infected DH82 cells treated with cytochalasin D from which the cell membrane had been removed. (**g**) Transmission electron micrograph of *E. muris*-infected DH82 cell treated with cytochalasin D. *Thick arrows* indicate *Ehrlichia* morulae, N, nucleus. Scale bar, 1 μm. (**h**) A single IOE cell in mouse spleen. *Arrows* indicate actin filaments

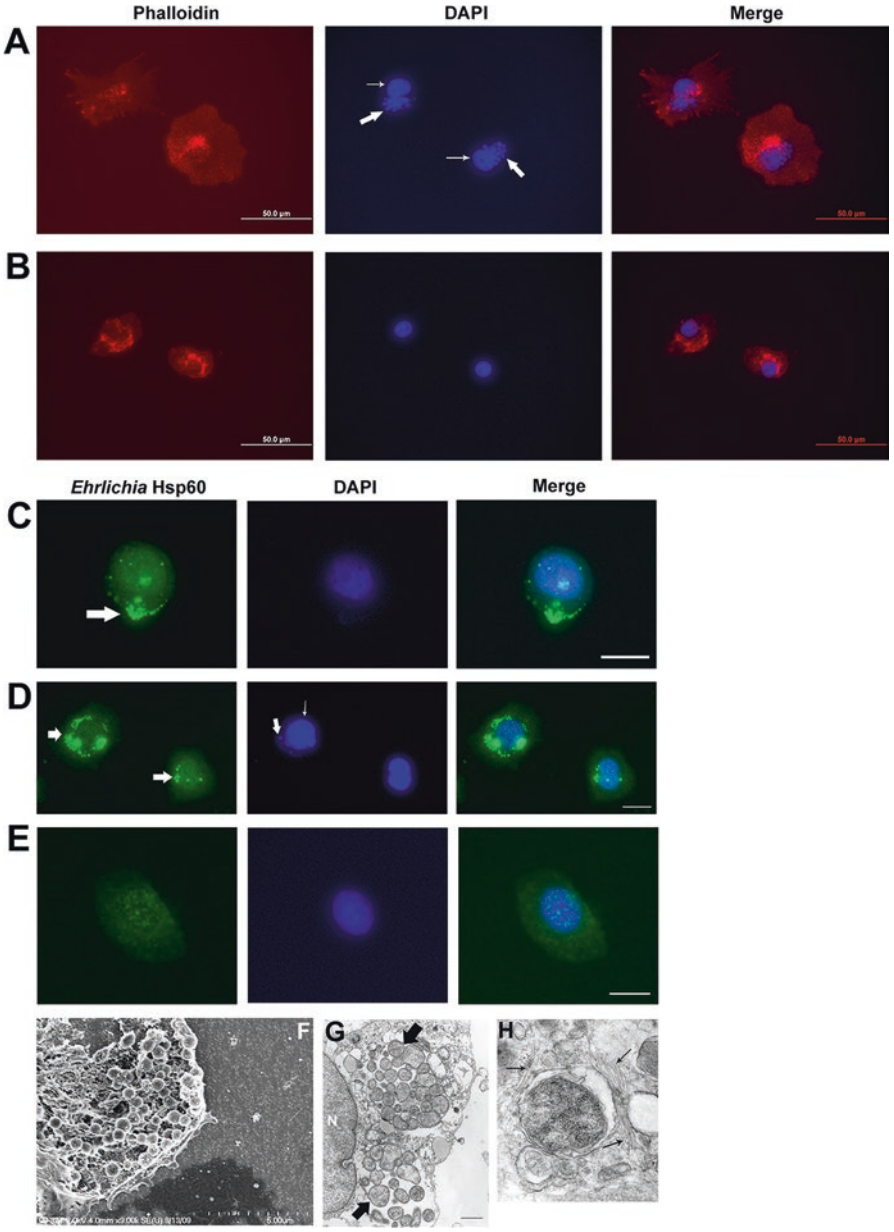

Fig. 8.12 Cytochalasin D inhibited filopodium formation in *Ehrlichia*-infected cells. (**a**) E. chaffeensis-infected DH82 cells treated with cytochalasin D and stained with phalloidin (*left*), DAPI (*middle*) (*thick arrows* indicate *Ehrlichia* morulae and *thin arrows* indicate host nuclei), and merged figure (*right*). (**b**) Uninfected DH82 cells treated with cytochalasin D and stained with phalloidin. (**c**, **d**) E. muris-infected DH82 cells treated with cytochalasin D and probed with *Ehrlichia* Hsp60 antibody (*left*) (*thick arrow* indicates *Ehrlichia*), DAPI (*middle*) (*thick arrow* indicates *Ehrlichia* DNA, and *thin arrow* indicates host cell nuclei), and merged figure (*right*).

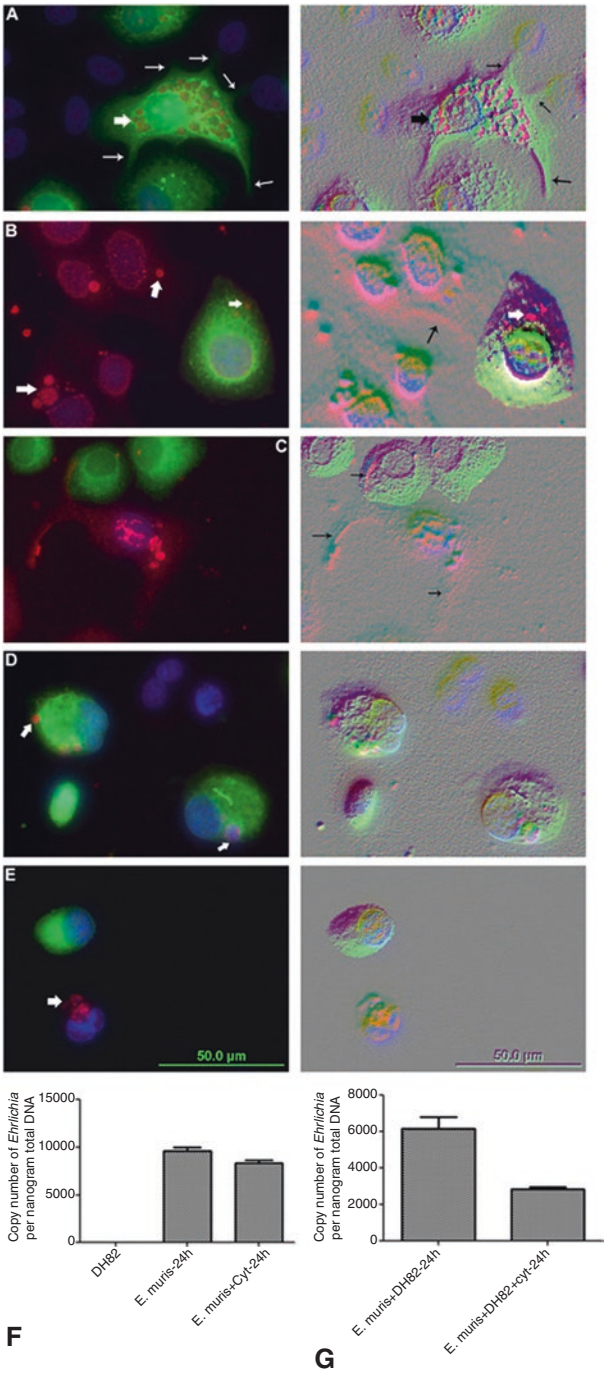

Fig. 8.13 Inhibition of filopodium formation prevented *Ehrlichia* intercellular transport. (**a**) *Ehrlichia*-infected DH82 cells were treated with CFSE and seeded with uninfected non-treated DH82 cells for 24 h. *Thick arrow* indicates *E. muris* (probed with *Ehrlichia* Hsp60), whereas the *thin arrows* indicate filopodia/pseudopodia of infected cells. DAPI stains the nucleus of both the

5 *Ehrlichia*-Induced Filopodia in Infected Mouse Macrophages

It is difficult to observe *Ehrlichia*-induced filopodium formation in monocytes/macrophages in vivo. Hence, splenocytes of mice that had been infected with *Ehrlichia* were cultured for 7 days. The cytoplasm of the macrophages from *E. muris*-infected mice harbored few pathogens on days 1–3 in cell culture, whereas by day 5 the macrophages were highly populated with *E. muris* (Fig. 8.14a, c–f, m). *E. muris*-infected mouse macrophages had filopodia that contained the pathogen similar to those observed in the infected DH82 cells. Similar results were observed when macrophages from the highly lethal *Ixodes ovatus* Ehrlichia (IOE)-infected mice (infected for 7 days prior to harvesting) were cultured for 5 days in vitro. IOE were also observed in the filopodia of infected mouse macrophages (Fig. 8.14b, g, h).

6 In Late Stages of Infection the *Ehrlichia* Ruptured the Overlying Host Cell Membrane

When DH82 cells infected with *E. muris* were cultured for 60 h, the morula was found to be enlarged probably due to fusion of adjacent morulae (Fig. 8.15b; Morulae of *E. muris* at 24 h: Fig. 8.15a). The cell membrane of *E. muris*-infected DH82 cell ruptured at 60 h (Fig. 8.15c) and the bacteria were released through the pores on the host cell membrane (Fig. 8.15d). The pathogens released after membrane rupture were observed attached to the filopodium of neighboring cells (Fig. 8.15e). Attached ehrlichiae were observed in association with ruffled cell membrane characteristic of entry by endocytosis (Fig. 8.15f). TEM of IOE-infected

Fig. 8.13 (continued) uninfected and infected cells. The adjacent figure is the Nomarski image, which clearly showed the filopodia/pseudopodia of infected cells. (**b**, **c**) DH82 cells were treated with CFSE and seeded with infected non-treated DH82 cells for 24 h. *Thick arrow* indicates *E. muris* (probed with *Ehrlichia* Hsp60) whereas the *thin arrows* indicate filopodia/pseudopodia of infected cells. DAPI stains the nuclei of both uninfected and infected cells. The adjacent figure is the Nomarski image which showed clearly the filopodia/pseudopodia of infected cells. (**d**) Ehrlichia-infected DH82 cells were treated with CFSE and seeded with uninfected non-treated DH82 cells for 24 h in the presence of cytochalasin D. *Thick arrow* indicates *E. muris* (the adjacent figure is the Nomarski image). (**e**) Uninfected DH82 cells were treated with CFSE and seeded with infected non-treated DH82 cells for 24 h in the presence of cytochalasin D. *Thick arrow* indicates *E. muris* (the adjacent figure is the Nomarski image). (**f**). Quantitative real-time PCR of bacterial loads of *E. muris*-infected DH82 cells to evaluate cytotoxicity in the presence of cytochalasin D ($n = 3$ per group). (**g**). Quantitative real-time PCR of bacterial load of *E. muris*-infected DH82 cells seeded with uninfected DH82 cells in the presence and absence of cytochalasin D ($n = 3$ per group)

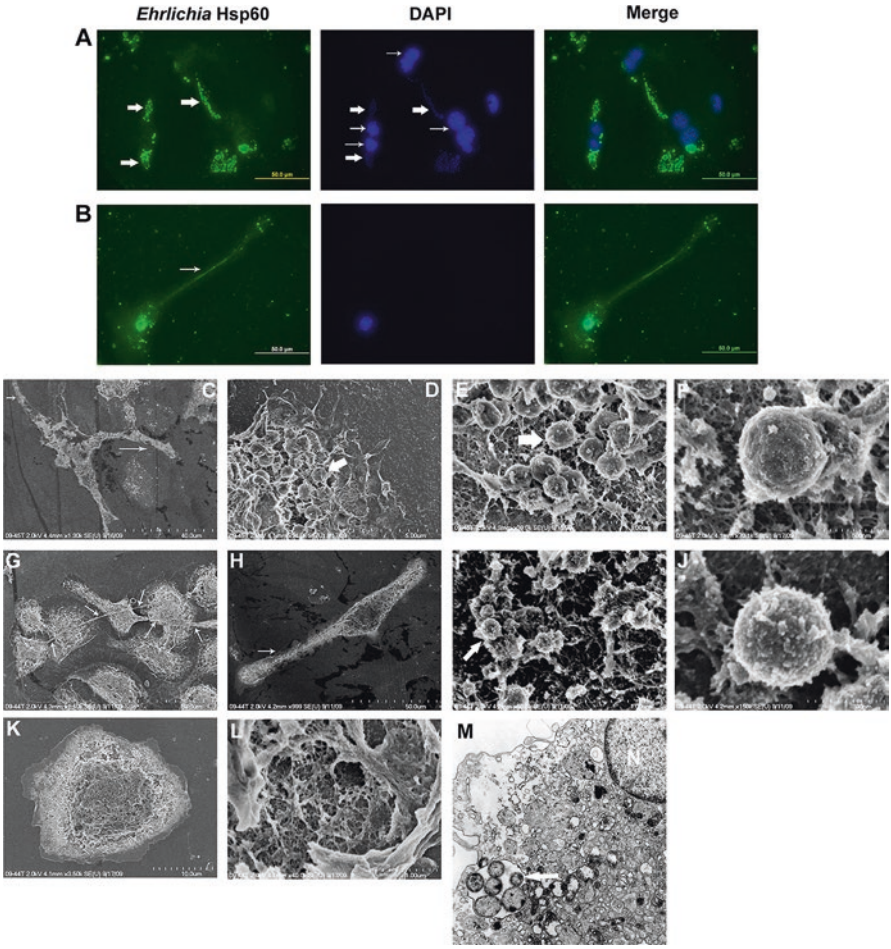

Fig. 8.14 *Ehrlichia* are observed in the filopodia of mouse macrophages. (**a**) *E. muris*-infected mouse macrophages probed with *Ehrlichia* Hsp60 antibody (*left*), DAPI (*middle*) (*thick arrows* indicate DNA of *E. muris*, and *thin arrows* indicate mouse macrophage nuclei), and merged figure (*right*). (**b**) IOE-infected mouse macrophage probed with *Ehrlichia* Hsp60 antibody (*left*) (*thin arrow* indicates filopodium), DAPI (*middle*), and merged figure (*right*). (**c**) Scanning electron micrograph of *E. muris*-induced filopodium in a mouse macrophage; *thin arrow* indicates the filopodium. (**d**) The interior of a mouse macrophage from which the cell membrane has been removed contained *E. muris*. (**e**) Higher magnification of *E. muris* in a mouse macrophage. (**f**) Scanning electron micrograph of an *E. muris* bacterium. (**g**, **h**) Scanning electron micrograph of IOE-induced filopodia in mouse macrophages; *thin arrows* indicate the filopodia. (**i**) IOE microorganisms in a mouse macrophage. (**j**) Scanning electron micrograph of a single IOE bacterium. (**k**) Uninfected mouse macrophage. (**l**) High magnification of an opened uninfected mouse macrophage. (**m**) Transmission electron micrograph of a mouse macrophage that contained an *E. muris* morula (*thick arrow*), N, nucleus. Scale bar, 1 μm

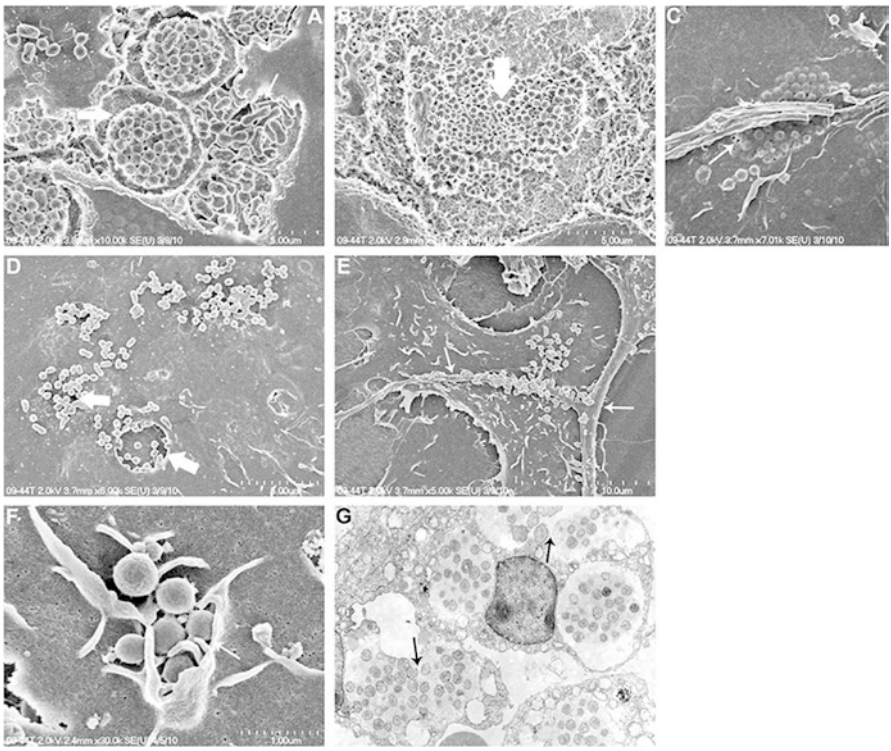

Fig. 8.15 *Ehrlichia* morulae inside a DH82 cell with an overlying ruptured host cell membrane. (**a**) Different stages of *E. muris* in a mechanically opened DH82 cell. *Thin arrow* indicates dividing ehrlichiae; *thick arrow* indicates mature cells. (**b**) Mature ehrlichiae cells in a large morula (*thick arrow*). (**c**) Pore formation on a DH82 host cell containing many ehrlichiae that have deformed the overlying cell membrane (*thin arrows*) (intact DH82 cell). (**d**) Host cell membrane ruptured at the location of ehrlichial exit from the cell (intact DH82 cell). (**e**) Extracellular *Ehrlichia* attached with high affinity to the filopodium of neighboring host cells (*thin arrows*). (**f**) Ehrlichiae attached to the DH82 cell membrane adjacent to a cell membrane ruffle (*thin arrow*) (intact DH82 cell). (**g**) TEM of an IOE-infected spleen (*arrows* indicate fused morula)

spleen confirmed morula fusion (Fig. 8.15g). Thus at a later time of infection, when the host cells are filled with the pathogenic bacteria, the *Ehrlichia* could disrupt the host cell membrane and exit the host cells.

7 Summary

Ehrlichia are obligatory intracellular bacterium which infects macrophages and monocytes. Using in vitro cell culture systems, the exit mechanism of *Ehrlichia* was described recently. The schematic diagram of *Ehrlichia* exit is shown in Fig. 8.16. *Ehrlichia* survive and replicate exclusively within inclusions in

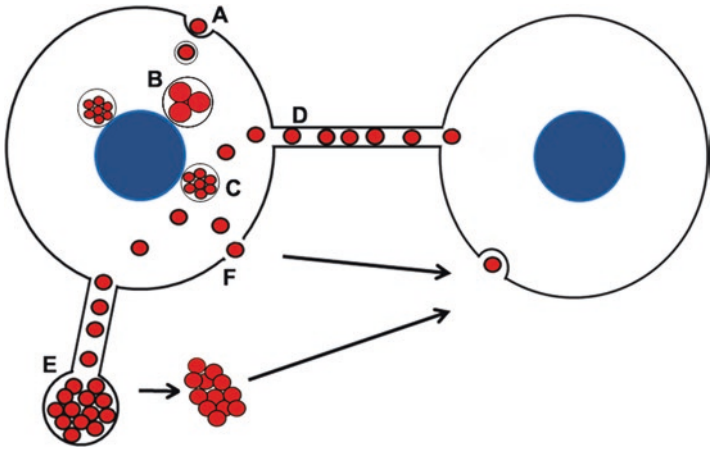

Fig. 8.16 Schematic diagram of *Ehrlichia* exit. *Ehrlichia* replicate and survive in host cell membrane-bound endosome compartment (A). The single cell *Ehrlichia* divides and form large pleomorphic reticulate cells (B). The reticulate cells divide and form the dense cored cells (C). The dense cored cells induce filopodium formation in host cells. The filopodium attach to neighboring cells and the pathogen pass through the filopodium to the neighboring cells (D). If there are no neighboring cells in the immediate vicinity, the end of the filopodium forms a fan shaped structure filled with *Ehrlichia* (E). These fan-shaped structures could detach and the cluster of ehrlichial cells could infect neighboring host cells. When the host cells are filled with the ehrlichial pathogens, they could break open the host cell membrane and the bacterial cells released (F)

monocytes and macrophages, which are primary effector cells of antimicrobial defense. Therefore, ehrlichiae must convert the hostile inclusion environment to a hospitable environment conducive to their survival and replication. Once the pathogenic bacteria are taken up, *Ehrlichia* replicate and survive in host cell membrane-bound endosome compartment (A). The *Ehrlichia* containing endosome does not fuse with the lysosome, thereby escaping destruction (Barnewall et al. 1997). The single cell *Ehrlichia* divides and form large pleomorphic reticulate cells (B). The reticulate cells divide and form the dense cored cells (C). Once the dense cored cells leave the morula, they could induce filopodium formation in host cells. The filopodium attach to neighboring cells and the pathogen pass through the filopodium to the neighboring cells (D). If there are no neighboring cells in the immediate vicinity, the end of the filopodium forms a fan-shaped structure filled with *Ehrlichia* (E). These fan-shaped structures could detach later and the cluster of ehrlichial cells could infect neighboring host cells. When the host cells are filled with the ehrlichial pathogens, they could break open the host cell membrane and the bacterial cells released (F).

8 Note to Readers

How the exit strategy of *Ehrlichia* was observed for the first time

Every scientific project is like a Russian doll (matryoshka doll); there is a hidden component lurking somewhere in the project that the investigator has to discover. Most often, the new discovery or phenomenon (the hidden component(s)) will turn out to be greater or groundbreaking than the planned main project. The important characters required for the investigator to discover something novel includes: a keen sense of observation, creativity, and imagination.

While working on *Ehrlichia* at the University of Texas Medical Branch, Galveston, Texas, my major goal was to develop a vaccine for *Ehrlichia*. The first question I had to address was why *Ehrlichia muris* is non-virulent compared to the highly virulent *Ixodes ovatus* ehrlichia (IOE). Both the strains had the same protein profile when characterized by SDS-PAGE (1D or 2D gels) or after probing with *Ehrlichia* antibodies by Western blotting. Prior to working on *Ehrlichia*, I used to work on lipid rafts at Mount Sinai School of Medicine, New York. We used to detect lipoproteins on T cells with Cholera toxin B (CTB); hence, I wondered whether it was possible to detect the lipoproteins of *Ehrlichia* by CTB on a blot. When the transferred proteins of *Ehrlichia* were probed with CTB, I observed some of the antigenic proteins in *E. muris* to be more lipoylated. Subsequently, I asked whether it was possible to observe glycosylated proteins. Since lectins bind to glucose moieties, I used wheat germ agglutinin (WGA) and concanavalin A (ConA) as probes. Though WGA was not a good probe, nevertheless, ConA could detect the glycosylated antigenic proteins of *Ehrlichia*. Finally, phosphomolybdate was used to detect phosphorylated proteins of *Ehrlichia*. The antigenic proteins of *E. muris* (GroEL/*Ehrlichia* Hsp60 and P28-19) had more post-translational protein modifications compared to the antigenic proteins of IOE. I coined the term "Eastern Blotting" for the detection of post-translational protein modifications.

Using *in silico* analyses, we determined the epitopes of *Ehrlichia* Hsp60 and P28-19. We generated peptides to these epitopes and they were injected in mice and the antibody generated was used to diagnose *E. muris* in DH82 cells.

When culturing *Ehrlichia*, the procedure involves seeding *E. muris*-infected DH82 cells on 60–70 % confluent uninfected DH82 cells. After 1 week of culturing, the infection rate is determined by light microscopy (Diff-quik staining). At that period the DH82 cells are fully confluent (cells closely packed) and it is difficult to observe any single cells. As I was not interested in highly infected DH82 cells, I seeded around 1000 DH82 cells infected with various amounts of *E. muris* on a culture slide and after 16 h stained with the newly generated antibodies. After 16 h of culture, the DH82 cells were far apart, and to my amazement I could observe tail like extensions (filopodia) on the host cells; some of the filopodia were seen attached to the neighboring cells. On staining with the *Ehrlichia* Hsp60 antibody, I could observe *E. muris* in the cells (in the cytoplasm as well as in the filopodia). Later, Dr. Vsevolod Popov showed me how to "peel open" the DH82 cells with Scotch tape and he asked his technician Julie Wen to help me with the staining for scanning

electron microscope (SEM). Since the laboratory was busy, Dr. Popov encouraged me to work on the SEM. While working on the SEM, I observed that after *Ehrlichia* are over-populated inside host cells, they break open the host cell membrane and exit cells. These exited cells could return and infect the neighboring cells. The findings were very well received by the scientific community.

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Chapter 9
Development of Vaccines for Ehrlichiosis

Sunil Thomas

1 Introduction

The obligate intracellular bacterium *Ehrlichia chaffeensis* that resides in mononuclear phagocytes is the etiologic agent of human monocytotropic ehrlichiosis (HME). HME is an emerging and often life-threatening zoonotic, tick-transmitted infectious disease in the United States (Paddock and Childs 2003; Thomas et al. 2010). Lack of early diagnosis and treatment of HME are the main factors that lead to severe and fatal disease. *Ehrlichia* also causes diseases in companion animals and domesticated ruminants. *E. chaffeensis* and *E. canis* cause canine ehrlichioses in dogs, whereas *E. ruminantium* causes heartwater in cattle, sheep, and goats. Vaccines are required for these tick-transmitted pathogens, but are hindered by many obstacles that exist in their development. These include knowledge of genetic and antigenic variability, identification of the ehrlichial antigens that stimulate protective immunity or elicit immunopathology, development of animal models that reflect the immune responses of the hosts and understanding molecular host–pathogen interactions involved in immune evasion or that may be blocked by the host immune response. Until recently, several strains of ehrlichiae were observed only in animals. However, recent studies demonstrated that many strains of *Ehrlichia* earlier confined to animals are now also observed to infect humans.

In April 1986, a medical intern scanning the peripheral blood smear of a severely ill man with an unexplained illness observed peculiar intracytoplasmic inclusions in several of the patient's monocytes. The patient-described multiple tick bites sustained approximately 2 weeks earlier during a visit to a rural area in northern Arkansas. The disease was initially diagnosed as Rocky Mountain spotted fever.

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Clinicians and scientists subsequently identified these inclusions as clusters of bacteria belonging to the genus *Ehrlichia* (it was initially called *E. canis*), previously known in the United States solely as veterinary pathogens (Maeda et al. 1987). The sequence comparisons indicated that the human ehrlichiosis agent was a new species most closely related to *E. canis* (98.2%) and more distantly related to other *Ehrlichia* spp. Anderson et al. (1991) proposed that this species be named *Ehrlichia chaffeensis* with the Arkansas strain as the type strain. Thus *E. chaffeensis* was the first *Ehrlichia* described as a human pathogen in 1986 (Paddock and Childs 2003). Later, *E. ewingii* (Buller et al. 1999) and *E. muris*-like agents (Pritt et al. 2011), pathogens associated with animals, were observed to infect humans. As yet there are no commercially available vaccines to protect against ehrlichiosis in humans and animals (Thomas et al. 2011, 2016). There is a lack of interest by commercial entities to develop vaccines for ehrlichiosis as there are several strains of *Ehrlichia*. Hence, there is a need to develop novel vaccines that protect against multiple strains of *Ehrlichia*.

Vaccines are considered as one of the most successful medical intervention against infectious diseases. Vaccines include killed or attenuated organisms or purified products derived from them. One of the drawbacks of killed or attenuated vaccines is the potential side effect of some of the antigenic proteins. This led to the design of recombinant vaccines based on whole antigens. As whole antigenic proteins are not essential in inducing immunity, it led to the emergence of a new branch of vaccine design termed "structural vaccinology" (Dormitzer et al. 2008; Nuccitelli et al. 2011). Structure-based vaccines have the rationale that protective epitopes are enough to induce immune responses and provide protection against pathogens (Koide et al. 2005). Structure-based peptide antigens induce antibodies which recognize the denatured form of a protein from which their sequences are derived (Rowlands 1992). It is difficult to predict the strategy to develop an efficacious vaccine; hence, multiple strategies have to be tested to develop an efficacious vaccine for ehrlichiosis.

2 Antigenic Proteins of *Ehrlichia*

The *Ehrlichia* species that are associated with human or veterinary diseases include: *E. chaffeensis*, *E. ewingii*, *E. canis*, *E. ruminantium*, *Ixodes ovatus* ehrlichia, *E. muris*, and *E. muris-like agent*. Development of a murine model of persistent ehrlichiosis has greatly facilitated our understanding of the pathogenesis and mechanisms of host defenses against ehrlichial infections. Mildly virulent *Ehrlichia muris* infection in mice results in persistent infection and mimics *E. chaffeensis* infection in its natural host, white-tailed deer (Olano et al. 2004). Murine models of systemic infection associated with the mildly virulent *E. muris* or the highly virulent IOE (*Ixodes ovatus Ehrlichia*) have provided knowledge of immunological mechanisms involved in host defenses against ehrlichial infection (Sotomayor et al. 2001; Bitsaktsis et al. 2004; Olano et al. 2004). Protective immunity against ehrlichiae involves both humoral and cell-mediated immunity.

The ehrlichial antigenic proteins are selected for vaccines based on reactivity of the proteins with antibodies from sera of infected animals. This is based on the rationale that immunoreactive proteins induce antibodies which could protect against bacterial pathogens. The major immunoreactive proteins of *E. chaffeensis* include 200-, 120-, 88-, 55-, 47-, 40-, 28-, 23-, and 19-kDa proteins (Luo et al. 2008). Analysis of immunoreactive antigens for peak intensity and relative quantity identified major immunoreactive *E. canis* antigens recognized early in the infection as the 19-, 37-, 75-, and 140-kDa proteins. Later in infection, additional major immunoreactive *E. canis* proteins were identified, including the 28-, 47-, and 95-kDa proteins (McBride et al. 2003).

The major antigenic proteins of *Ehrlichia* are outer membrane proteins (OMP-1/P28) encoded by a multi-gene family, gp19, p29, gp36, gp140, gp200, ferric ion-binding protein (Fbp), disulfide bond formation (Dsb) protein, GroEL (*Ehrlichia* Hsp60), MAP2, VLPT, ankyrin repeat proteins, and tandem repeat proteins (TRP) (Alleman et al. 2001; Cárdenas et al. 2007; Luo et al. 2008; Thomas et al. 2011; Thirumalapura et al. 2013). Ortholog tandem repeat proteins of *E. chaffeensis* and *E. canis*, TRP120/TRP140, TRP75/TRP95, TRP47/TRP36, and TRP32/TRP19 contain major antibody epitopes in the tandem repeat regions (Luo et al. 2008, 2009; Luo and McBride 2012; McBride et al. 2011). Ehrlichial TRPs are secreted, serine/threonine-rich, and acidic, which results in higher electrophoretic mobility than their predicted molecular masses (Wakeel et al. 2009). The TRPs contain varying numbers of tandem repeats in different ehrlichial species and strains. Host cell proteins that are targeted by TRPs include proteins involved in signaling, vesicle trafficking, and transcriptional regulation. The interactions between TRPs and host targets cause the redistribution of some host proteins to ehrlichial morula or cytoplasm adjacent to the morulae in *E. chaffeensis*-infected cells, further indicating the profound effects of TRPs on host cell protein recruitment (Wakeel et al. 2009; Luo et al. 2011; Luo and McBride 2012).

Ankyrin repeat protein orthologs (p200) are large 200-kDa proteins that have been characterized in *E. chaffeensis* and *E. canis* (Zhu et al. 2009). The p200 target genes for tumor necrosis factor alpha, Stat1, and CD48 and these are strongly upregulated during ehrlichial infection.

The outer membrane proteins (OMPs) of *Ehrlichia* play a crucial role in virulence and pathogenesis. These antigenic proteins are immunoreactive and aids in vaccine development (Moumène et al. 2015). The chaperone protein GroEL (Hsp60) is found on the outer membrane and plays a role in the folding of a large number of proteins; they are also involved in bacterial adhesion (Kusukawa et al. 1989).

The strongly acidic 19-kDa major immunoreactive glycoprotein (gp19) elicits an early *Ehrlichia*-specific antibody response in infected dogs. *E. canis* gp19 has substantial carboxyl-terminal amino acid homology (59 %) with *E. chaffeensis* VLPT and the same chromosomal location; however, the *E. chaffeensis* VLPT gene (594 bp) has tandem repeats that are not present in the *E. canis* gp19 gene (414 bp). *E. canis* gp19 composition consists of five predominant amino acids, cysteine, glutamate, tyrosine, serine, and threonine, concentrated in the STE-rich patch and a carboxyl-terminal domain predominated by cysteine and tyrosine (55 %).

The amino-terminal STE-rich patch contained a major species-specific antibody epitope strongly recognized by serum from an *E. canis*-infected dog. The gp19 protein is present on reticulate and dense-cored cells and is localized predominantly in the cytoplasm of ehrlichiae; it is also found extracellularly in the fibrillar matrix and associated with the morula membrane (McBride et al. 2007).

All members of *Rickettsiales* have limited biosynthetic capabilities due to the loss of genes required by free-living bacteria during reductive genome evolution (Dunning Hotopp et al. 2006). These bacteria, therefore, cannot survive extracellularly and are obliged to import most nutrients and metabolic products from their host cells. In order for small hydrophilic compounds, such as sugars, amino acids, or ions to pass through, the outer membrane of Gram-negative bacteria have β-barrel proteins called porins that function as passive diffusion channels (Kumagai et al. 2008). The immunodominant P28/OMP-1 family of proteins is the most abundant outer membrane proteins in *E. chaffeensis* (Ohashi et al. 1998). Different alleles from this multigene family are expressed in different host cell types. P28 family members are the most studied *E. chaffeensis* outer membrane proteins (OMPs). They have multiple predicted transmembrane β strands and are encoded by an antigenically variant multigene family composed of 22 paralogous genes clustered in a 27-kb gene locus of the *E. chaffeensis* genome. All 21 *p28* genes of *E. muris* are transcriptionally active in vivo on day 9 post-infection in mice (Crocquet-Valdes et al. 2011). Differential expression of the p28 family of outer membrane genes (OMP-1) occurs, with OMP-1b (p28-14) upregulated in tick cells, and p28-19, an integral membrane protein with porin activity that is upregulated in mammalian cells (Kuriakose et al. 2013).

The outer membrane protein of *E. muris*, P29 is an ortholog of *E. chaffeensis* TRP47 and *E. canis* TRP36. Unlike *E. chaffeensis* TRP47 and *E. canis* TRP36, orthologs of *E. muris* (P29) and *E. muris*-like agent (EMLA) do not contain tandem repeats (Thirumalapura et al. 2013).

E. chaffeensis p120 is an outer membrane protein that is preferentially expressed on the dense-core ultrastructural form of *E. chaffeensis* but not on the reticular cell (Popov et al. 2000). The p120 is an adhesin of *E. chaffeensis* (Popov et al. 2000; Yu et al. 2000). p120 is expressed on the surface of the microorganism and free in the morula space; however, the role of this protein in pathobiology or in eliciting a protective immune response is unknown (Popov et al. 2000). *E. chaffeensis* p120 has two to five nearly identical serine-rich 80-amino-acid TRs; similarly, orthologous *E. canis* p140 contains 12 or 14 nearly identical serine-rich 36-amino-acid TRs. The TR regions of the p120 and p140 proteins are immunoreactive; however, the specific molecular immunodeterminant(s) is not defined (Luo et al. 2009). The glycoprotein genes of *Ehrlichia chaffeensis* (1644 bp) and *Ehrlichia canis* (2064 bp) encode proteins of 548–688 amino acids with predicted molecular masses of only 61 and 73 kDa but with electrophoretic mobilities of 120 kDa (P120) and 140 kDa (P140), respectively. The 120-kDa protein gene of *E. chaffeensis* contains four identical 240-bp tandem repeat units, and the 140-kDa protein gene of *E. canis* has 14 nearly identical, tandemly arranged 108-bp repeat units. Antibodies against the recombi-

nant P120 and P140 proteins reacted with *E. chaffeensis* P120 and *E. canis* P140, respectively. Carbohydrate was detected on the *E. chaffeensis* and *E. canis* recombinant proteins, including the two-repeat polypeptide region of *E. chaffeensis* P120. A carbohydrate compositional analysis identified glucose, galactose, and xylose on the recombinant proteins. The presence of only one site for N-linked (Asn-Xaa-Ser/Thr) glycosylation, a lack of effect of *N*-glycosidase F, the presence of 70 and 126 Ser/Thr glycosylation sites in the repeat regions of P120 and P140, respectively, and a high molar ratio of carbohydrate to protein suggest that the glycans may be O linked (McBride et al. 2000).

gp200 is the largest major immunoreactive ehrlichial protein ortholog of *E. canis* and *E. chaffeensis* (Nethery et al. 2007). The native and recombinant *E. chaffeensis* and *E. canis* gp200 orthologs exhibit molecular masses larger than those predicted by their amino acid sequences but lack serine-rich tandem repeats present in other ehrlichial proteins (McBride et al. 2003). The gp200 is a secreted nuclear translocated ankyrin repeat-containing protein that numerous ankyrin repeats that may mediate protein–protein interactions. gp200 has five major species-specific epitopes that are primarily located in terminal acidic domains (Nethery et al. 2007). The protein has been shown to elicit strong antibody responses in the acute phase of the infection (Zhang et al. 2008). Though the function of gp200 is unknown, the protein is translocated to the nucleus of infected monocytes. gp200 exhibits homology with *Anaplasma phagocytophilum* AnkA, which is a type IV secretion substrate and is phosphorylated by host Abl-1 and Src tyrosine kinases (Caturegli et al. 2000; Lin et al. 2007). Initially, p43 was thought to be an antigenic protein of *E. canis* (McBride et al. 2001). Later studies demonstrated that *E. canis* p43 represents the N-terminal portion of the largest immunoreactive protein described in *Ehrlichia* spp. with a predicted molecular mass of 153 kDa (McBride et al. 2003). A native *E. canis* protein with a molecular mass of 200 kDa reacted with antisera produced against the N-terminal region (p43) of the p153, suggesting that the native protein was post-translationally modified. Similarly, recombinant constructs of *E. chaffeensis* p156 migrated larger than predicted (approximately 200 kDa), and carbohydrate was detected on the recombinant proteins. The chromosomal location, amino acid homology, and biophysical properties support the conclusion that the p153 and p156 glycoproteins (designated gp200s) are species-specific immunoreactive orthologs.

3 The Importance of Cellular and Humoral Immunity in Bacterial Clearance

Ehrlichia cause persistent infection in their natural hosts (e.g., *E. ruminantium* in certain ruminants and *E. chaffeensis* in white-tailed deer); whereas, infection of some accidental hosts results in a severe toxic shock-like illness (e.g., *E. chaffeensis* in humans and *Ixodes ovatus* ehrlichia [IOE] in experimentally inoculated mice)

(Feng and Walker 2004). Studies of the immune response to *E. chaffeensis*, *E. ruminantium*, and *E. canis* as well as the host response to *E. muris* and IOE have all contributed to the overall understanding of the cell-mediated and humoral host responses to *Ehrlichia* spp. (Thomas 2016).

The importance of antibodies in the control of *E. canis* was demonstrated initially by Lewis and colleagues in 1978 (Lewis et al. 1978; Lewis and Ristic 1978). The authors demonstrated that specific antibodies could inhibit the growth of *E. canis* in vitro. The importance of humoral immunity in ehrlichiosis was demonstrated when passive transfer of antibodies could prevent fatal disease during *Ehrlichia chaffeensis* infection of immunodeficient SCID mice (Winslow et al. 2000). Further studies have concluded that antibodies against specific p28 linear epitopes located in a hypervariable region mediate this protection (Li et al. 2001, 2002). The antibody-mediated bacterial clearance, at least in part, by opsonizing bacteria released from infected host cells (Li and Winslow 2003). These findings demonstrated a possible therapeutic role for antibodies during ehrlichial infections (Yager et al. 2005).

There are well documented studies on the importance of cell-mediated involvement in protective immunity. MyD88-dependent signaling is required for controlling ehrlichial infection by playing an essential role in the immediate activation of the innate immune system and inflammatory cytokine production, as well as in the activation of the adaptive immune system at a later stage by providing for optimal Th1 immune responses (Koh et al. 2010). Numerous studies with multiple *Ehrlichia* spp. indicate that IFN-γ is an essential mediator of protection (Totté et al. 1993, 1994). Moreover, $CD4^+$ and $CD8^+$ T cells both contribute to IFN-γ production (Esteves et al. 2004). Notably, similar conclusions regarding the importance of MHC class I, $CD4^+$, and $CD8^+$ T cells, and the synergistic roles of IFN-γ and TNF-α have been reported in mice infected with *E. muris* (Feng and Walker 2004). An important role for $CD4^+$ T cells in immunity to *E. ruminantium* and IOE has been suggested (Totté et al. 1998; Bitsaktsis et al. 2004). Similarly, mice lacking functional MHC class II genes are unable to clear *E. chaffeensis* infection, suggesting that $CD4^+$ T cells are essential for ehrlichial clearance (Ganta et al. 2002, 2004). The route of administration of *Ehrlichia* influences cellular immunity. Stevenson et al. (2006) demonstrated that the intradermal environment (natural route of inoculation) appears to promote the induction of protective type-1 responses characterized by increased $CD4^+$ and $CD8^+$ T cells and IFN-γ producing $CD4^+$ T cells. However, B cells are also involved in immune responses against the pathogenic *Ehrlichia*.

Immunity to the highly pathogenic IOE revealed that B cells are essential for protection in immunocompetent mice following a low-dose sublethal infection (Yager et al. 2005). However, low-dose IOE-infected wild-type mice generated relatively poor antibody responses and were not protected from a subsequent fatal high-dose IOE challenge infection. In contrast, infection with the low pathogenic *E. muris* was shown to generate effective immunity to IOE challenge. Protection against high-dose IOE was mediated by B cells and antibodies and can be generated in the absence of CD4 T-cell-mediated help (Bitsaktsis et al. 2004). The studies

demonstrated the importance of antibodies in providing protection against *Ehrlichia* infection. Overall, studies demonstrated that both the host humoral and cell-mediated immunity is essential in the control of *Ehrlichia* bacteria during infection.

4 Development of Vaccines for Ehrlichiosis

Though several antigenic proteins of *Ehrlichia* are defined and characterized, there are only very few vaccines that have been generated based on these antigenic proteins. Vaccines that are reported for ehrlichiosis are based on the antigenic proteins *Ehrlichia* Hsp60 (GroEL), P28-9, P28-12, P28-19, and P29. There are no vaccines based on *Ehrlichia* TRPs; however, it has been reported that passive transfer of antibodies against *E. chaffeensis* TRPs provides protection against challenge in mice (Kuriakose et al. 2012). As yet there are no effective vaccines available commercially, either for humans or animals, and all of them are in the preclinical stages of development.

4.1 Attenuated Strain of E. ruminantium *as Vaccines*

Heartwater, the tick-borne disease of ruminants, is caused by the intracellular *Ehrlichia ruminantium*. Current immunization procedure involves infecting animals with cryopreserved sheep blood containing virulent *E. ruminantium* organisms, followed by treatment with tetracyclines when fever develops. Attenuated strain of *E. ruminantium* is also used as an immunogen. The virulent Welgevonden strain of *E. ruminantium* was attenuated by continuous propagation of the organisms in DH82 cell line, followed by re-adaptation to grow in a bovine endothelial cell line, BA 886. Sheep and goats inoculated with the attenuated organisms followed by lethal needle challenge with the virulent homologous stock were protected (Zweygarth et al. 2005). A study in Burkina Faso demonstrated that immunization of sheep with the Gardel strain of *E. ruminantium* was more efficacious compared to other strains in providing protection against heartwater (Adakal et al. 2010).

4.2 The Outer Membrane Protein P28 as a Vaccine Candidate

OMPs are immunodominant B-cell antigens and that passive transfer of anti-OMP antibodies can protect mice from fatal ehrlichial infection. Nandi et al. (2007) demonstrated that recombinant P28-19 (OMP 19) of IOE could elicit strong humoral and cellular responses in mice and they induced significant protection against lethal challenge.

Use of DNA vaccines is another strategy to provide protection against infectious diseases. A naked-DNA vaccine based on *p28* was found to protect mice against challenge with a lethal dose of *Ehrlichia ruminantium* (Nyika et al. 1998). Vaccination strategies using pathogen DNA priming followed by administration of homologous recombinant proteins have demonstrated enhanced immune responses compared with vaccines using DNA vaccination alone. Our group had recently used a DNA-prime/protein boost vaccination strategy to control *Ehrlichia* infection (Crocquet-Valdes et al. 2011). In the DNA-prime/protein boost vaccination strategy, the initial immunization is with DNA and the final immunization with the corresponding protein. C57BL/6 mice were immunized with recombinant DNA plasmids carrying the *p28-9*, *p28-12*, *p28-14*, and *p28-19* genes or a mixture of all four in combination with IL-12 DNA on days 0 and 28 followed by two homologous recombinant protein booster immunizations with P28-9, P28-12, P28-19, and the P28 mixture days 56 and 84. IL-12 is known to enhance cellular immunity. Combining DNA vaccine with immune stimulatory molecules delivered as genes (IL-12) significantly enhance Ag-specific immune responses in vivo (Sin et al. 1999). Immunized mice were challenged intraperitoneally with a high dose of *E. muris* 28 days after the last booster immunization, and the bacterial burden in the spleen was determined on day 7 after challenge. A high bacterial burden was detected in the spleens of mice immunized with empty vector/protein (mock-vaccinated mice). In contrast, the spleens of mice vaccinated with P28-9, P28-12, P28-19, and the P28 mixture exhibited significantly reduced bacterial loads on day 7 post-infection than those of the mock-vaccinated control group (Crocquet-Valdes et al. 2011).

As the P28-19 DNA/protein immunization was found to reduce the ehrlichial burden significantly, we further evaluated recombinant P28-19 (rP28-19) as a vaccine candidate. We immunized mice with two doses of rP28-19 (15 days apart) and challenged them with *E. muris* 15 days after the last dose of immunization. We assayed the bacterial burden in the spleens and livers of mice vaccinated with the P28-19 protein and unvaccinated mice harvested on days 7 and 14 after *E. muris* challenge by quantitative real-time PCR. The spleens of rP28-19-vaccinated mice had significantly lower bacterial loads on day 7 than the spleens of unvaccinated mice. Furthermore, on day 14, there were no detectable bacteria by qPCR in either the spleens or livers of the rP28-19-vaccinated mice (Crocquet-Valdes et al. 2011).

Analysis of P28-19-specific antibody responses by ELISA on days 7, 14, and 21 after *E. muris* challenge demonstrated that the IgG response in vaccinated mice challenged with *E. muris* was highest on day 14 compared to day 7 or 21. We further analyzed the isotypes of P28-19-specific antibodies in sera from mice. Both vaccinated and unvaccinated mice challenged with *E. muris* had higher concentrations of P28-19-specific IgM and IgG2b antibodies on day 7 after the challenge. The rP28-19-vaccinated mice challenged with *E. muris* had substantially higher levels of IgG1, IgG2b, and IgG3 antibodies on day 14 after *E. muris* challenge than mice infected with *E. muris* alone. In contrast, mice infected with *E. muris* alone developed substantial levels of P28-19-specific IgG2c, IgG2b, and IgG3 by day 21 post-infection.

To determine cellular immune response to the vaccine, we determined by flow cytometry whether P28-19-specific T cells are induced during *E. muris* infection. Splenocytes from *E. muris*-infected mice were harvested on day 45 post-infection

and stimulated in vitro with the recombinant P28-19 protein for 18 h. Compared to uninfected naïve mice, *E. muris*-infected mice had significantly higher frequencies of P28-19-specific IFN-γ-producing $CD4^+$ Th1 cells in their spleens and they develop into memory cells (Crocquet-Valdes et al. 2011).

4.3 P29 as a Vaccine Candidate

Our group had evaluated the efficacy of recombinant *E. muris* P29 (an ortholog of *E. chaffeensis* TRP47 and *E. canis* TRP36), as a subunit vaccine candidate (Thirumalapura et al. 2013). Immunization with recombinant *E. muris* P29 conferred significant protection against challenge infection. Mice were immunized with recombinant *E. muris* P29 proteins in the presence of adjuvant by the i.p. route, followed by a booster immunization 30 days after primary immunization. The immunized and control mice were challenged with the *E. muris* 60 days after the booster immunization.

Mice immunized with rP29 had significantly reduced bacterial loads in the liver, spleen, lung, and blood after the *E. muris* challenge infection compared to naïve unimmunized control mice. Mice immunized with the rP29 had high levels of rP29-specific IgG antibodies in sera before and after *E. muris* challenge. Examination of immunoglobulin isotypes indicated that mice immunized with rP29 developed high levels of anti-rP29 serum IgG1, IgG2c and IgG2b, and low concentrations of IgG3 antibodies. In contrast, immune sera collected from mice after primary or secondary *E. muris* infection had high concentrations of IgG2c and lower concentration of IgG2b and IgG3 with no detectable concentration of IgG1 directed against the *E. muris*-lysate antigen as determined by ELISA.

To determine cellular immunity, the splenocytes from mice infected with *E. muris* at day 30 post-infection were stimulated in vitro with rP29 or *E. muris* lysate antigen for 24 h. Flow cytometric analysis indicated that *E. muris*-infected mice had significantly higher frequencies of P29-specific CD4+ Th1 cells in the spleen compared to naïve uninfected mice. CD4+ T cells target P29 during *E. muris* infection and differentiate into IFN-γ-producing Th1 effector/memory cells. CD4+ Th1 cells mediate immune responses against intracellular pathogens, and IFN-γ produced by CD4+ Th1 cells could activate macrophages and enhance their microbicidal activity (Thirumalapura et al. 2013).

4.4 Development of Structure-Based Vaccines for Ehrlichiosis

The genetic diversity of microorganisms, coupled with the high degree of sequence variability in antigenic proteins, presents a challenge to developing broadly effective conventional vaccines. The observation that whole protein antigens are not necessarily essential for inducing immunity has led to the

emergence of a new branch of vaccine design termed "structural vaccinology". Structural vaccinology combines elements of structural biology and bioinformatics into a promising new method for the identification of antigenic protein elements of interest based on the protein amino acid sequence and the resulting secondary and tertiary structure. The enabling principle is that the entire antigenic protein is not essential for inducing an immune response as only the epitope sequence per se actually induces the immune response and provide protection against pathogens. Recent studies demonstrated that designing structure-based vaccine candidates with multiple epitopes induce a higher immune response. Structural vaccinology is quickly emerging as a viable strategy for the rational design of vaccine candidates because structure-based vaccines based on epitopes appear to be more specific, inherently safer and easier and less costly to produce (Thomas and Luxon 2013).

Analysis of *E. muris* splenocyte lysate by polyclonal antibodies from *E. muris*-infected mice demonstrated *Ehrlichia* Hsp60 (GroEL) and OMP-1 (P28) as the major antigenic protein of *E. muris* (Thomas et al. 2009). Using eastern blotting, we determined that these proteins are also post-translationally modified. We used the probes concanavalin A, Cholera Toxin B, and nitrophospho–molybdate complex for the detection of glucose, lipid, and phosphate residues, respectively (Thomas et al. 2009).

To determine a protein sequence for potential antigenic epitopes, sequences that are hydrophilic, surface-oriented, and flexible are selected. Most naturally occurring proteins in aqueous solutions have their hydrophilic residues on the protein surface and hydrophobic residues buried in the interior. Three regions of the *E.* muris P28-19 and Hsp60 protein sequence had good hydrophilicity predicted by the Lasergene software (DNAStar, WI, USA). The hydrophilic sequences of both the *Ehrlichia* P28-19 and Hsp60 proteins with no hydrophobic residues were selected. The hydrophilic regions of P28-19 correspond to amino acids 55–75, 91–103, and 124–145 (Fig. 9.1). The hydrophilic regions of *Ehrlichia* Hsp60 correspond to amino acids 43–63, 179–199, and 387–406 (Fig. 9.2). The sequences showed homology to other *Ehrlichia* species. The peptides (underlined) were synthesized and conjugated to KLH and used as probes to detect antibodies to *E. canis* and *E. chaffeensis* or to raise antibodies.

The 3D structure of P28-19 in Fig. 9.1 was modeled using the online I-TASSER (iterative threading assembly refinement) server. The 3D structure of *Ehrlichia* Hsp60 in Fig. 9.2 was modeled using the online Phyre2 server (Thomas et al. 2011). The P28-19 $_{55–75}$ and *Ehrlichia* Hsp60 $_{43–63}$ epitope peptides induced antibodies (Thomas et al. 2010, 2011); hence, we reasoned that they also could provide protection against *Ehrlichia* thereby functioning as potential vaccine candidates.

Mice were immunized i.p., with two doses P28-19 $_{55–75}$ peptide or *Ehrlichia* Hsp60 $_{43–63}$ peptides conjugated to KLH 15 days apart (the first immunization with complete Freund's adjuvant and the second immunization with incomplete Freund's adjuvant). Thirty days after the first immunization, mice were challenged intraperitoneally (i.p.) with a high dose of *E. muris* ($\sim 1 \times 10^4$ bacterial genomes) and

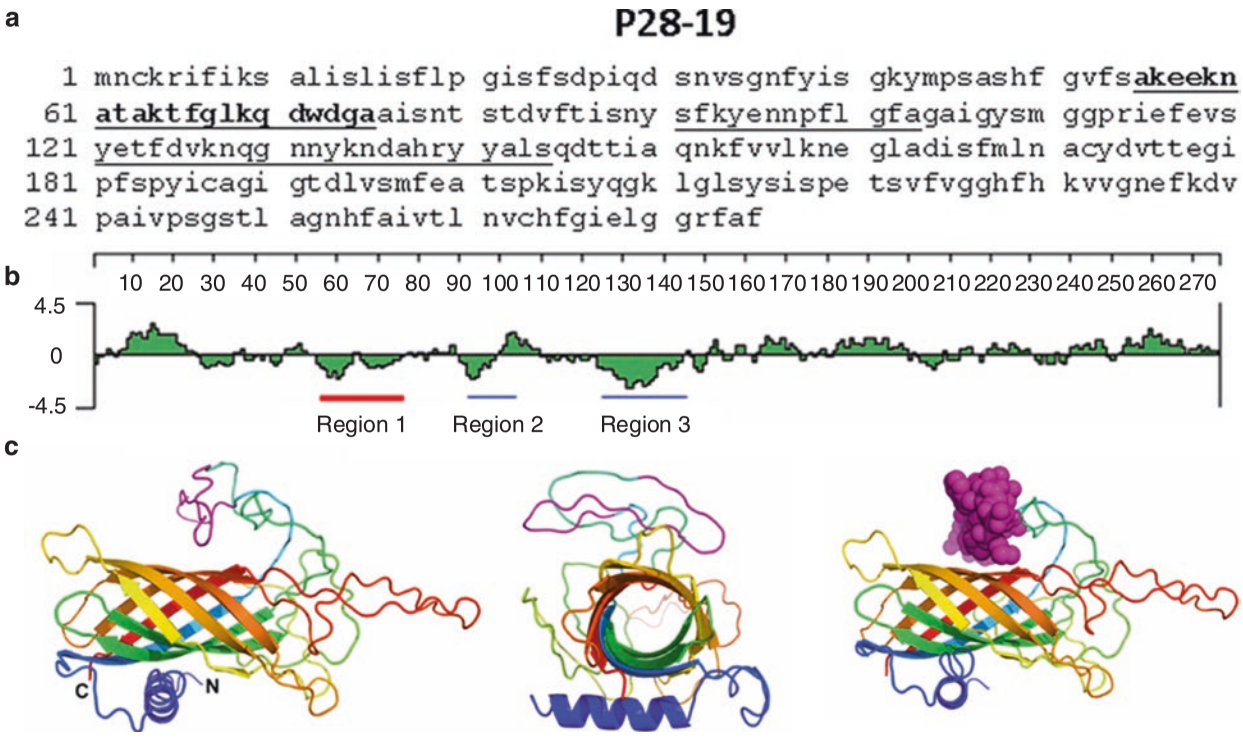

Fig. 9.1 Amino acid sequence of P28-19. (**a**) P28-19 peptides corresponding to the *underlined* predicted hydrophilic sequence were synthesized. The peptide corresponding to the *bold underlined* (55–75) sequence was found to react with antibodies to *Ehrlichia* as well as to induce antibody production. (**b**) Hydrophobicity plot of P28-19. The sequences *underlined* (in *red* and *blue*) were used for synthesizing peptides; however, the best peptide sequence selected is *underlined* in *red*. (**c**) (*Left*) Predicted 3D structure of P28-19 (side view), (*Middle*) predicted 3D structure of P28-19 (basal view), (*Right*) predicted 3D structure of P28-19 with the Van der Waals radii of the heavy atoms highlighting the region of interest (P28-19 $_{55–75}$)

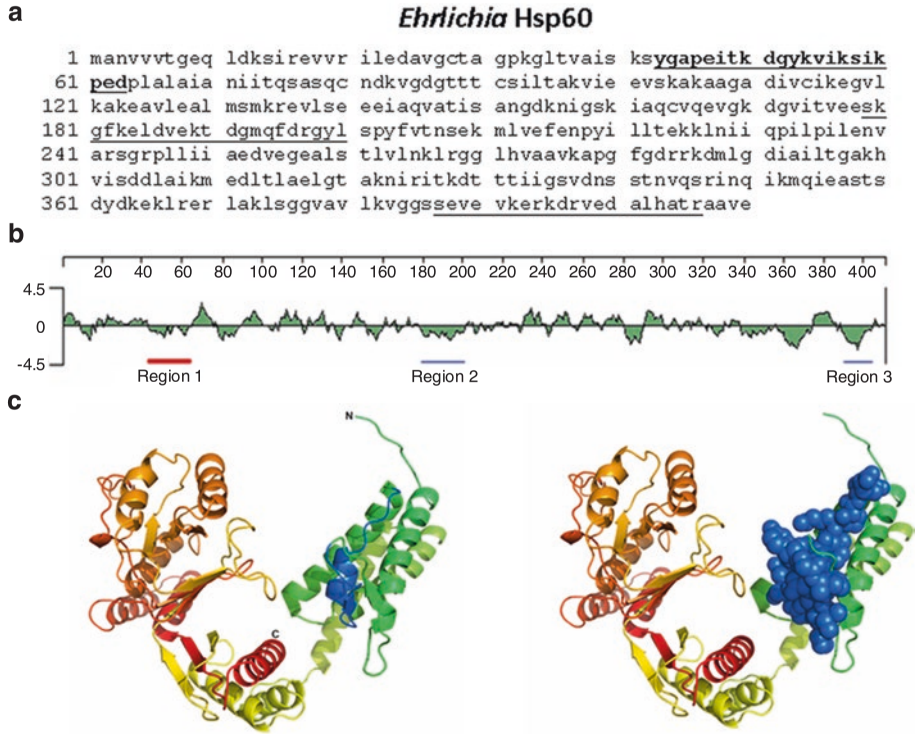

Fig. 9.2 Amino acid sequence of *Ehrlichia* Hsp60. (**a**) Hsp60 peptides corresponding to the *underlined* predicted hydrophilic sequence were synthesized. The peptide corresponding to the *bold underlined* (43–63) sequence was found to react with antibodies to *Ehrlichia* as well as to induce antibody production. (**b**) Hydrophobicity plot of *Ehrlichia* Hsp60. The sequences *underlined* (in *red* and *blue*) were used for synthesizing peptides; however, the best peptide sequence selected is *underlined* in *red*. (**c**) (*Left*) Predicted 3D structure of *Ehrlichia* Hsp60, (*Right*) predicted 3D structure of *Ehrlichia* Hsp60 with the Van der Waals radii of the heavy atoms highlighting the region of interest ($Hsp60_{43–63}$)

observed daily. Controls included unchallenged naïve mice as well as unvaccinated mice injected with *E. muris* alone. Mice were sacrificed on days 7, 14, and 21 after ehrlichial challenge, and spleen and liver were harvested and sera collected. The ehrlichial load in spleen and liver was determined by quantitative RT-PCR. Sera were assayed for determination of antibody titers.

We immunized mice with P28-19 $_{55–75}$ or *Ehrlichia* Hsp60 $_{43–63}$ epitope peptides and challenged 30 days later with *E. muris*. The spleen and liver were collected at different days after bacterial challenge and the bacterial copy number determined by quantitative real-time PCR. We observed lower bacterial load in both spleen and liver on days 7 and 14 after bacterial infection in the vaccinated mice compared to unvaccinated controls (Fig. 9.3). The data demonstrated that P28-19 $_{55–75}$ and *Ehrlichia* Hsp60 $_{43–63}$ peptides functioned as vaccine candidates and provided protection against *Ehrlichia* infection.

Immunization with vaccines stimulates the immune system to produce a robust antibody response that can provide protection against pathogens. To determine the antibody responses against the *Ehrlichia* Hsp60 $_{43–63}$ peptide vaccine, blood was collected from vaccinated mice on days 7 and 14 and performed ELISA. There was a significant difference in the antibody response between unvaccinated and *Ehrlichia* Hsp60 $_{43–63}$ vaccinated mice after challenge with *E. muris*. However, there was no difference between the antibody levels in vaccinated mice between days 7 and 14. The *Ehrlichia* Hsp60 $_{43–63}$-specific antibody levels in infected unvaccinated mice were highest on day 14 compared to day 7. To determine the antibody responses against the P28-19 $_{55–75}$ peptide vaccine, blood was collected from immunized mice on days 7 and 14 and subjected the samples to ELISA. There was a significant difference in the antibody response between unvaccinated and P28-19 $_{55–75}$ vaccinated mice after challenge with *E. muris*. Antibody levels were higher on day 14 compared to day 7 (Fig. 9.4).

As antibody isotype responses can be useful indicators of immune bias during infection, we determined the antibody isotypes after vaccination with the peptide epitopes. The level of antibody isotypes increased by day 14 compared to day 7 after bacterial challenge. The *Ehrlichia* Hsp60 $_{43–63}$-vaccinated mice had higher levels of IgG1, IgG2c, IgG2b, IgG3, and IgM after bacterial challenge compared to unvaccinated mice on day 14. By ELISA, we analyzed the isotypes of the antibodies of P28-19 peptide in vaccinated and unvaccinated mice after challenge with *E. muris* (day 14 post challenge). The P28-19 $_{55–75}$ vaccinated mice challenged with *E. muris* had higher levels of IgG1, IgG2b, IgG3, and IgM compared to unvaccinated mice infected with the pathogen.

Flow cytometry was used to determine whether *Ehrlichia* Hsp60 $_{43–63}$ and P28-19-specific memory T cells are induced during *E. muris* infection. Splenocytes from *E. muris*-infected mice were harvested on day 45 post-infection and stimulated in vitro with the *Ehrlichia* Hsp60 $_{43–63}$ and P28-19 $_{55–75}$ for 18 h. Compared to uninfected naïve mice, *E. muris*-infected mice had significantly higher frequencies and absolute numbers of *Ehrlichia* Hsp60 $_{43–63}$ and P28-19 $_{55–75}$-specific IFN-γ-producing CD4+ Th1 cells in their spleen (Fig. 9.5). As both the antigens *Ehrlichia* Hsp60 as well as P28-19 could induce B cells and T cells, our studies conclude that both the antigenic proteins are highly efficient vaccine candidates to protect against *Ehrlichia* infection.

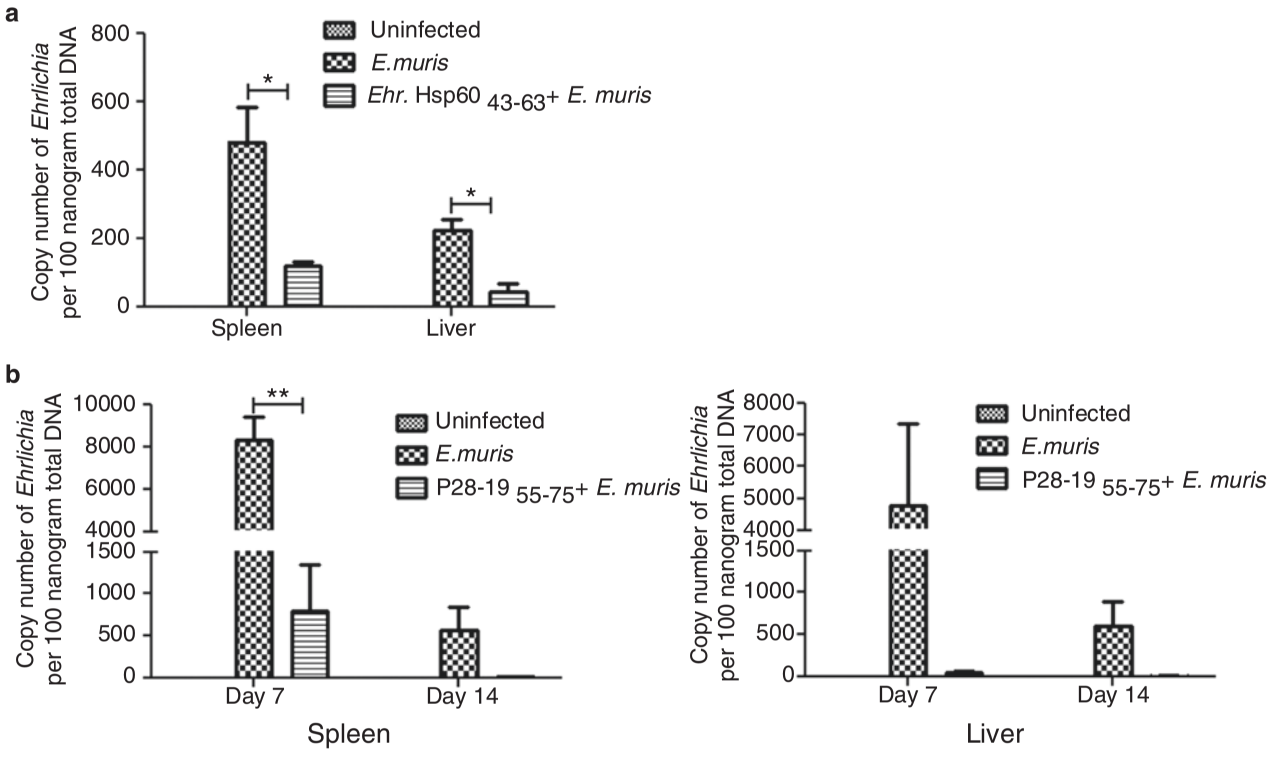

Fig. 9.3 Immunization with *Ehrlichia* Hsp60 $_{43–63}$ and P28-19 $_{55–75}$ peptides protected mice from *Ehrlichia* infection. (**a**) Mice immunized with *Ehrlichia* Hsp60$_{43–63}$ were protected against *E. muris* challenge as determined by the bacterial load measured by quantitative real-time PCR on day 14 after *E. muris* challenge (*$p<0.05$ as determined by *t* test). (**b**) Mice immunized with P28-19 $_{55–75}$ peptide was protected against *E. muris* challenge as determined by the bacterial load measured by quantitative real-time PCR on days 7 and 14 after *E. muris* challenge (**$p<0.01$ as determined by *t* test)

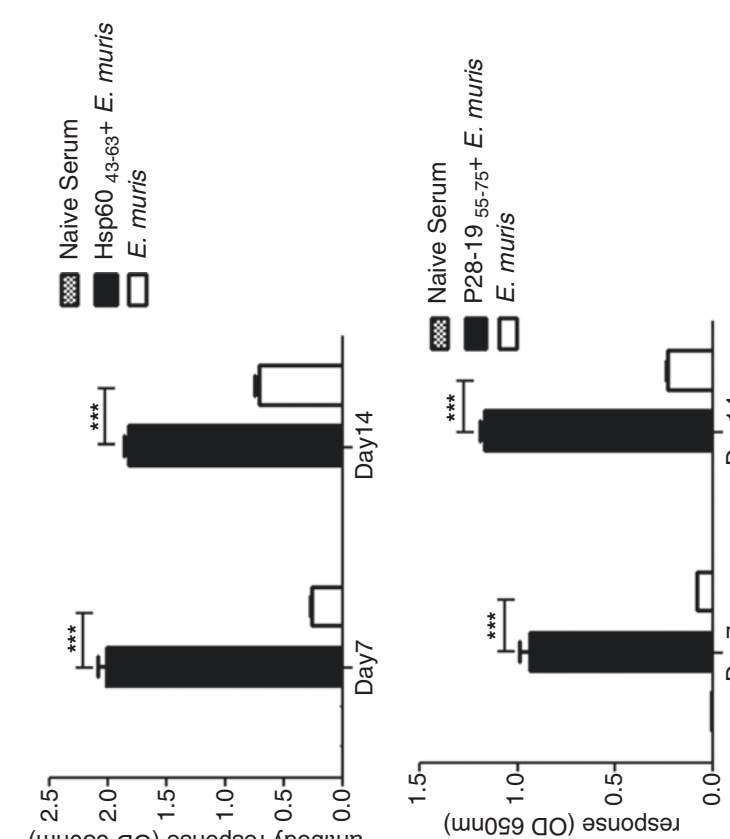

Fig. 9.4 Protection induced by *Ehrlichia* Hsp60 $_{43–63}$ and P28-19 $_{55–75}$ peptides was associated with induction of *Ehrlichia*-specific IgG antibody. (**a**) *Ehrlichia* Hsp60 $_{43–63}$ vaccinated mice induced higher IgG antibody levels after challenge with *E. muris* compared to unvaccinated *E. muris*-infected mice (***$p<0.001$ as determined by *t* test). (**b**) P28-19 $_{55–75}$ peptide vaccinated mice induced higher IgG antibody levels after *E. muris* challenge compared to unvaccinated *E. muris*-infected mice (***$p<0.001$ as determined by Student *t* test)

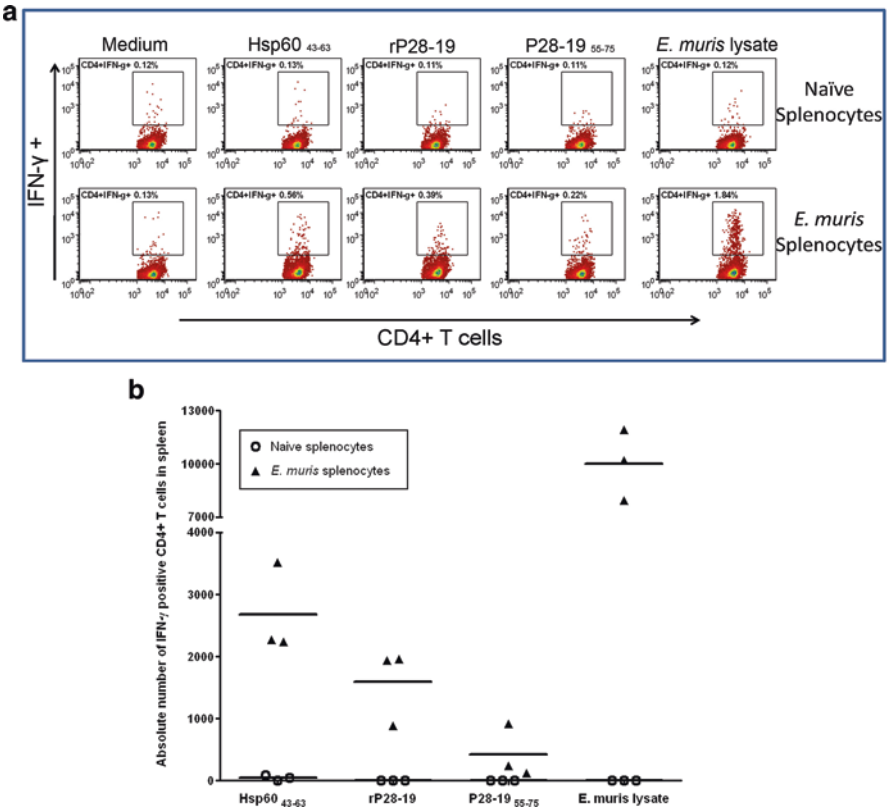

Fig. 9.5 *Ehrlichia* Hsp60 $_{43–63}$ and P28-19 $_{55–75}$-specific memory CD4+ T cells develop during *E. muris* infection. By flow cytometry, the frequencies and absolute numbers of *Ehrlichia* Hsp60 $_{43–63}$- and P28-19-specific IFN-γ-producing CD4+ T cells in the spleen of mice infected with *E. muris* was determined. (**a**) Mice infected with *E. muris* had higher frequency of *Ehrlichia* Hsp60 $_{43–63}$- and P28-19 $_{55–75}$-specific IFN-γ-producing CD4+ T cells in the spleen on day 45 after infection compared to naïve uninfected mice. Representative dot plots were gated on live cells followed by CD3+ T cells (**b**) Absolute numbers of *E. muris*-specific IFN-γ-producing CD4+ T cells in the spleen of the same mice detected following in vitro stimulation with the *Ehrlichia* Hsp60 $_{43–63}$, P28-19 $_{55–75}$ peptides; rP28-19 and *E. muris* whole cell lysate are shown for comparison. Horizontal bars represent the mean; data are representative of two independent experiments ($n = 3$ animals per group)

5 Conclusion

As yet there are no vaccines for the intracellular Gram-negative bacterium causing ehrlichiosis in animals and humans (Thomas et al. 2016). Though many antigenic proteins of *Ehrlichia* are described, as yet there are very few vaccine candidates even in preclinical stages. Another factor that is preventing a lack of interest by vaccine manufacturers in developing vaccines for *Ehrlichia* is the presence of several ehrlichial strains that infects animals and humans, which may require multiple

vaccines. Development of a recombinant or structure-based vaccine incorporating several antigenic proteins or epitopes may lead to the development of novel vaccines that protect against multiple strains of *Ehrlichia*.

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Chapter 10
Identification of T-Cell Epitopes in the Murine Host Response to *Ehrlichia chaffeensis*

Stephen K. Chapes, M. Teresa Ortega, Chuanmin Cheng, and Roman R. Ganta

The genus *Ehrlichia* contains a number of species that infect humans and several other mammals through a tick bite. The complex host requirements for tick development, the number of species that the bacteria successfully infect (Paddock and Childs 2003), and the variations in the different *Ehrlichia* species complicate the complete understanding of the host immune response to these organisms (Chapes and Ganta 2008). For example, *Ixodes ovatus Ehrlichia* (IOE) is a highly pathogenic species and pathogenicity depends on the route of infection (Stevenson et al. 2006). *E. muris* causes a persistent infection in mice, and exposure to this organism can induce protection against IOE. In contrast, *E. chaffeensis* does not protect against IOE and is not considered "cross protective" (Ismail et al. 2004). Therefore, as with other *Rickettsia* (Riley et al. 2015), some *Ehrlichia* antigen epitopes may be more immunogenic and induce protective immunity than others.

E. chaffeensis, which causes human monocytic ehrlichiosis (Paddock et al. 1997), expresses an array of outer membrane proteins depending on the parasitized host (Singu et al. 2005, 2006; Seo et al. 2008). *Ehrlichia* gene expression can affect host range (Cheng et al. 2015) either by affecting the properties of the paracitoferous vacuole (Cheng et al. 2014), by regulating the molecules it uses to bind to target cells (Mohan Kumar et al. 2013) or by other undefined mechanisms. Human monocytic ehrlichiosis was formally recognized in the late 1980s; it probably was misdiagnosed previous to that (Doudier et al. 2010). Moreover, a combination of better awareness and better diagnostic tools have resulted in an increase in the documented incidence of infections with species of *Ehrlichia* and *Anaplasma* since 2000 (Dahlgren et al. 2011).

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The outer membrane proteins and their predominant expression of a 28 kDa outer membrane protein, p28-OMP 14, when the bacterium grows in tick cells to the predominant expression of a related 28 kDa outer membrane protein, p28-OMP 19, when it grows in mammalian cells may challenge the host immune system to mount an efficient response to clear the infection (Ganta et al. 2007). However, Crocquet-Valdes et al. found that multiple Ehrlichial p28 outer membrane isoforms were able to induce immunity against *E. muris*, so this may not always be problematic (Crocquet-Valdes et al. 2011). Molecules that are necessary for *Ehrlichia* growth intracellularly, like tandem repeat proteins (Luo and McBride 2012) also can induce protective immunity (Kuriakose et al. 2012; Luo et al. 2008; Wakeel et al. 2010).

E. chaffeensis infection is cleared in approximately 2 weeks in healthy, immunocompetent mice (Ganta et al. 2004; Winslow et al. 2000). The rapid clearance is similar in healthy humans as infections with *E. chaffeensis* are milder in this group of people compared to elderly and immuno-compromised adults. T cells contribute to host resistance because in the absence of $CD4^+$ T cells or interferon-γ, there is a delay in bacterial clearance or diminished *Ehrlichia* resistance (Bitsaktsis et al. 2004; Ganta et al. 2004, 2007; Ismail et al. 2007). Previous studies have shown that Tlr4 plays some role in host resistance to *E. chaffeensis* (Ganta et al. 2002) and that MyD88 signaling in T cells may be necessary for the production of interferon-γ which may be necessary to activate monocytes and macrophages (Ganta et al. 2002; Chapes and Ganta 2008; Miura et al. 2011) and to regulate other host defense processes including the induction of hematopoiesis (Zhang et al. 2013). An *Ehrlichia* penicillin-binding protein may be responsible for cellular activation through MyD88 (Rahman et al. 2012).

Antibodies have been shown convincingly to provide protection independently of cell-mediated immunity (Li et al. 2002; Racine et al. 2011; Winslow et al. 2000, 2003). Components such as B-cell activating factor are important to the secretion of T-cell-independent antibody (Jones et al. 2013). Interestingly, many of the B cells responsible for protective immunity against *E. muris* were localized to the omentum (Jones et al. 2015). These B cells may be part of the natural antibody network compared to the B cells that are isolated from spleen which produce IgGs and are induced after immunization (Ganta et al. 2002, 2004, 2007).

Although it appears that animals can generate a vigorous immune response against *E. chaffeensis* infections, there are still some questions about the relative contributions of different components of the immune system. Whereas $CD4^+$ T cells have been found to be beneficial to host immunity during *Ehrlichia* infections (Ganta et al. 2004; Ismail et al. 2007; Nandi et al. 2007), the beneficial role of these cells is not so obvious (Bitsaktsis et al. 2004; Bitsaktsis and Winslow 2006; Feng and Walker 2004; Ismail et al. 2004). Nandi et al. described several T-cell idiotypes generated after mouse challenge with IOE p28-OMP 19 (Nandi et al. 2007). However, we recently demonstrated that *E. chaffeensis* differentially expresses p28-OMPs which is dependent on the host-cell environment (Singu et al. 2005, 2006), complicating the generation of host immunity because of differences in p28 OMPs (Ganta et al. 2007).

We previously demonstrated that effector memory was generated regardless of whether *E. chaffeensis* was propagated in DH82 macrophage cells or ISE6 tick cells (Ganta et al. 2007). Therefore, we have been interested in the properties of the host response after experimental challenge with *E chaffeensis*. In particular, we were interested in the T-cell epitopes that are capable of activating T cells and whether these epitopes were conserved between p28-OMP 14 and p28-OMP 19. Therefore, we defined $CD4^+$ and $CD8^+$ T-cell *E. chaffeensis* P28 OMP epitopes.

We have found that clearance of *E. chaffeensis* is delayed in the absence of helper T cells (Ganta et al. 2002, 2004). However, it is not clear what role $CD8^+$ T cells play in the host response to the organism. $CD8^+$ T cells appear to be detrimental during infection by other *Ehrlichia* species (Bitsaktsis et al. 2004; Feng and Walker 2004; Ismail et al. 2004; Bitsaktsis and Winslow 2006). Therefore, we examined the kinetics of *E. chaffeensis* clearance at the site of injection in CD8 knock-out mice and compared the clearance patterns to mice deficient for $CD4^+$ T cells.

As seen previously, there was a statistically significant delay in the clearance of bacteria in CD4 knock-out mice at the site of injection 13–15 days after experimental challenge (Fig. 10.1). When we compared CD8 knock-out mice to wild-

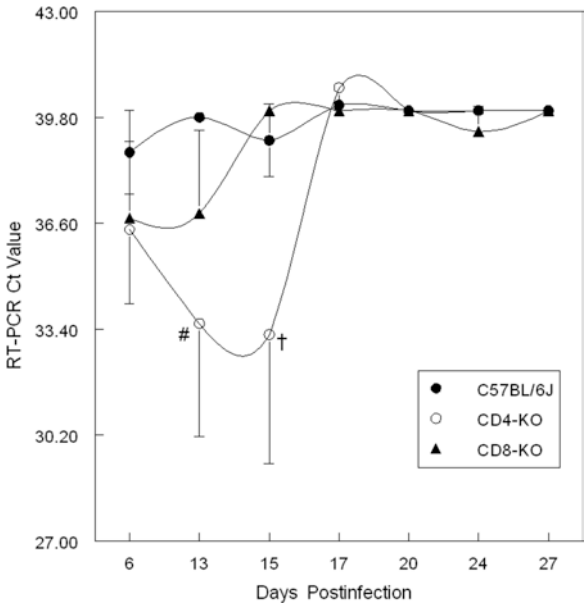

Fig. 10.1 Kinetics of B6, CD4 knock-out and CD8 knock-out mouse infections after i.p. experimental challenge with *E. chaffeensis* grown in DH82. *E. chaffeensis* clearance from the peritoneum (site of injection) was assessed by a 16S rRNA-based real-time RT-PCR assay. CT values of 40 represent clearance. The data represent the mean values ± SD of four to eight mice per time point per treatment group. # indicates significantly different from wild-type B6 mice, $P < 0.05$; † indicates different from wild-type B6 mice $P = 0.07$. The absence of an error bar means that the error is less than the size of the figure marker

type B6 mice, there was a small lag in clearance but those differences were not significant ($P = 0.07$) in the peritoneum (Fig. 10.1) or in any other tissues (data not shown).

T-cell activation is a hallmark of a successful host defense against *Ehrlichia* challenge (Bitsaktsis et al. 2004; Ganta et al. 2004, 2007; Ismail et al. 2007). Effective mobilization of effector memory cells after immunization with immunogenic *E. muris* has been associated with protective immunity against IOE (Thirumalapura et al. 2008). We previously found that mice challenged with *E. chaffeensis* mobilized effector memory T cells (Ganta et al. 2007). Effector memory T cells produce cytokines (effector activity) (Lai et al. 2011) and have low levels of CD62L and higher levels of CD44 (Sallusto et al. 1999). We found that there was effective mobilization of effector memory cells after both primary and secondary experimental challenges with *E. chaffeensis* (Fig. 10.2). In spite of this effective T-cell response to *E. chaffeensis* infection, little is known about the T-cell epitopes. Nandi et al. (2007) have shown that IOE p28-OMP 19-specific T cells are capable of protecting mice against IOE infection. They identified immunogenic IOE-specific

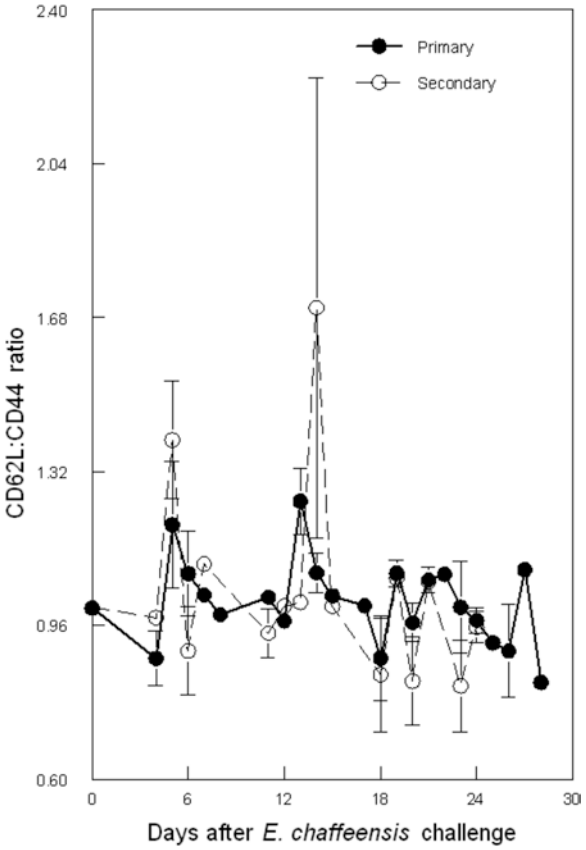

Fig. 10.2 Evaluation of CD62L:CD44 ratio of spleen lymphocytes after primary (*black circle*) or secondary (*white circle*) *i.p.* experimental challenge with *E. chaffeensis* grown in DH82 cells. The ratios of infected mice were normalized by dividing the same ratio found in uninfected mouse controls. A value of 1 is considered the value found in normal, uninfected mice. Numbers represent mean ± SD of independent samples evaluated from four to six mice per time point

T-cell epitopes by synthesizing a panel of overlapping 16 amino acid peptides that induced protection against IOE. Several of those epitopes are conserved in *E. chaffeensis* p28-OMP 19 (Nandi et al. 2007). Therefore, we synthesized three *E. chaffeensis* p28-OMP 19 peptides that would be homologous to IOE peptides 30–45 (W1), 107–122 (W2), and 197–212 (W3) (Nandi et al. 2007) (Table 10.1, Fig. 10.3). We also attempted to identify additional *E. chaffeensis* p28-OMP 19 peptides that would activate $CD4^+$ T cells. Predicting IA-binding peptides ($CD4^+$ T cell epitopes) is difficult. They can have irregular lengths and there is a poorer understanding of the rules governing MHCII anchor residues compared to MHCI (Brusic et al. 1998). However, the RankPep algorithm (http://bio.dfci.harvard.edu/Tools/rankpep.html) can predict nine amino acid MHCII-binding peptide cores along with three flanking amino acids at both ends (Reche et al. 2004). Using this method, we predicted three additional 15 amino acid IA^b-binding peptides for *E. chaffeensis* p28-OMP 19 (Table 10.1). The amino acids in the fourth/fifth positions in two of the peptides are aromatics: Phe, Tyr, or Try. Interestingly, there was not a complete overlap of our predicted peptides with *E. chaffeensis* peptides that were synthesized based on IOE (Fig. 10.3). Because the open ends of MHCII allow for longer peptide binding and there could be some flexibility in the epitope (Brown et al. 1993), we examined the ability of these six peptides to stimulate T-cell proliferation in vitro after primary or secondary in vivo challenges with *E. chaffeensis* organisms (Fig. 10.3). This is a sensitive assay that would allow us to determine whether these epitopes were immunogenic (Goodell et al. 2007). We confirmed that all three of the *E. chaffeensis* peptides that were based on IOE peptides (Nandi et al. 2007) were stimulatory. Peptide W2 stimulated the highest proliferative responses and peptide W1 stimulated high responses with spleen cells taken from animals challenged multiple time with *Ehrlichia* (Fig. 10.4). Of the peptides predicted by RankPep program, the VSMFEATNPKISY (C3) peptide was the most stimulatory and the EVSYETFDVKNQGNN (C2) peptide was the second most biologically active. The ISGKYMPSASHFGVF (C1) peptide was less biologically active after priming with whole bacteria than all the other peptides tested (Fig. 10.4).

We previously demonstrated that cytotoxic T lymphocytes (CTL) are not effectively induced to kill *Ehrlichia*-infected cells unless mice are given multiple bacterial challenges (Ganta et al. 2004). However, CD4 knock-out mice appear to clear bacteria faster the second time they are experimentally challenged with *E. chaffeensis* (Ganta et al. 2004). Because there is some debate about the possible pathogenic nature of $CD8^+$ T cells during some *Ehrlichia* infections, and the unknown nature of the $CD8^+$ T-cell response, we were interested in the $CD8^+$ T-cell idiotypes that were induced after *E. chaffeensis* challenge. Using the SYFPEITHI algorithm (http://www.syfpeithi.de/Scripts/MHCServer.dll/EpitopePrediction.htm), five peptides (eight amino acid lengths) from p28-OMP 19 were identified. The peptides were synthesized and incorporated into pentamers (Proimmune, Oxford, GB) (Fig. 10.5). In addition, we identified four p28-OMP 14 peptides which were also caged in pentamers to identify whether T cells specific for those epitopes were induced in response to bacteria grown in DH82 cells (Table 10.1 and Fig. 10.5). Spleen cells were collected various times after either a primary or secondary challenge with

Table 10.1 Predicted MHCI H-2K[b] and MHCII IA[b] binding peptides

H-2K[b] binding peptides[a]		IA[b] binding peptides[b]	
OMP-p28-14	OMP-p28-19	OMP-p28-19	*E. chaffeensis* peptides based on IOE sequence[c]
ADKKKYVVL (#6)	EAHRYCAL (#1)[d]	ISGKYMPSASHFGVF (C1)	GSGINGNFYISGKYMP (W1)
EGITFMSL (#7)	ASNNFVFL (#2)	EVSYETFDVKNQGNN (C2)	IGYSMDGPRIELEVSY (W2)
IGNNFNKI (#8)	IGNEFRDI (#3)	VSMFEATNPKISYQG (C3)	TDLVSMFEATNPKISY (W3)
IGGYYHGV (#9)	IGGHFHKV (#4)		
	SNYSFKYE (#5)		

[a]Peptides caged in pentamers for CD8$^+$ T-cell assessments. Predicted with the SYFPEITHI algorithm as described in the Materials and Methods

[b]Synthetic MHCII peptides predicted with RankPep algorithm and synthesized to stimulate *E. chaffeensis*-specific T-cell proliferation

[c]Homologous *E. chaffeensis* peptides synthesized based on immunogenic IOE peptides described by Nandi et al.

[d]Numbers in parenthesis represent peptides described in Fig. 10.7

[1]mnykkvfitsalislisslpgvsfsdpagsgingnfy***isgkympsashfgvf***sakeernttvgvfgl*kqnwdgsaisnsspn*
[2]mny----------*isgkymp*----------
[3]mnykkvfitsalislisslpgvsfsdpa***gsgingnfyisgkymp***sashfgvfsakeernttvgvfglkqnwdgsaisnsspn

dvftvsnysfkyennpflgfagaigysmdgpriel***evsyetfdvknqgnn***yks*eahrycalshnsaad*mssasnnfvflkn
----------*evsy*----------
dvftvsnysfkyennpflgfaga***igysmdgprielevsy***etfdvknqgnnykseahrycalshnsaadmssasnnfvflkn

eglldisfmlnacydvvgegipfspyicagigtdl***vsmfeatnpkisyqg***klglsysispeasvfigghfhkvi*gnefrdiptii*
----------*vsmfeatnpkisy*----------
eglldisfmlnacydvvgegipfspyicagig***tdlvsmfeatnpkisy***qgklglsysispeasvfigghfhkvignefrdiptii

*ptgs*tlaekgnypaivildvchfgielggrfaf
----
ptgstlaekgnypaivildvchfgielggrfaf

[1]Peptides (underlined and italicized) predicted to bind to IAb by the RankPep algorithym. Peptides used in these experiments (bold).

[2]Overlap (underlined and italicized) between RankPep predictions and those synthesized based on homology to IOE [17].

[3]Immunogenic peptides (underlined and italicized) based on homology to IOE [17].

Fig. 10.3 Binding of Ehrlichia p28-OMP 19 to IA[b]. [1]Peptides (*underlined* and *italicized*) predicted to bind to IA[b] by the RankPep algorithm. Peptides used in these experiments (*bold*). [2]Overlap (*underlined* and *italicized*) between RankPep predictions and those synthesized based on homology to IOE (Nandi et al. 2007). [3]Immunogenic peptides (*underlined* and *italicized*) based on homology to IOE (Nandi et al. 2007)

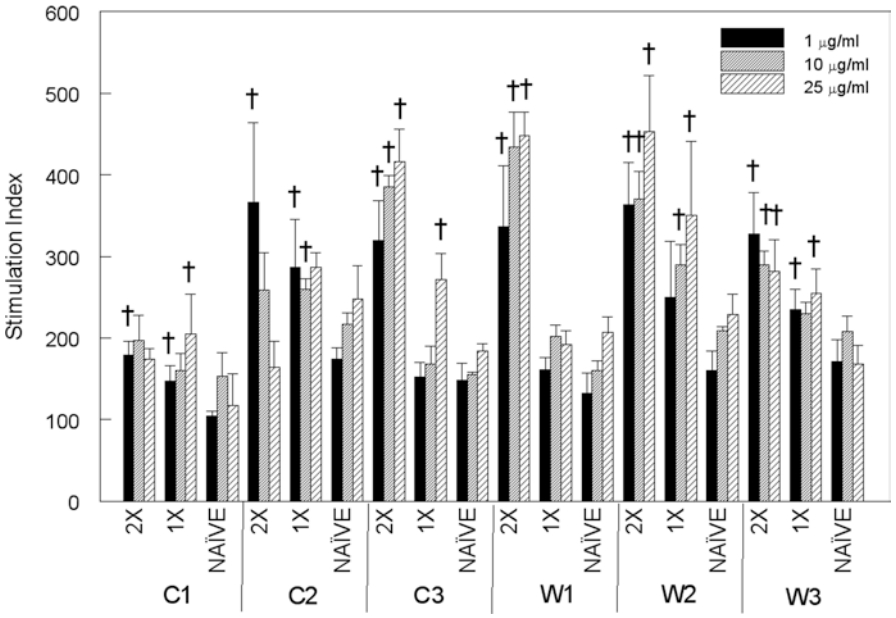

Fig. 10.4 Stimulation of T-cell proliferation using *E. chaffeensis* p28-OMP 19-derived peptides. Normal or mice previously challenged with *E. chaffeensis* grown in DH82 cells (1° or 2°) were incubated in vitro with peptides described in Fig. 10.3 (1, 10, and 25 μg/mL) for 5 days. Proliferation was assessed with [^{3}H]-thymidine as detailed in the Materials and Methods. Numbers represent mean ± SD of triplicate samples. † indicates significantly different from cells from naïve mice stimulated with the same peptide concentration

```
1   MNYKKVFITSALISLISSLPGVSFSDPAGS------GINGNFYISGKYMPSASHFGVFSAKEE-----RNTTVGVFGLKQ   69
1   MNYKKIFVSSALISLMSILPYQSFADPVTSNDTGINDSREGFYISVKYNPSISHFRKFSAEEAPINGNTSITKKVFGLKK   80

                                  5                                  *-------------------------*
70  NWDGSAISNSSPNDVFTVSNYSFKYENNPFLGFAGAIGYSMDGPRIELEVSYETFDVKNQGNNYKS--EAHRYCALSHNS  147
81  DGDIAQSAN----------FNRTDPALEFQNNLISGFSGSIGYAMDGPRIELEAAYQKFDAKNPDNNDTNSGDYYKYFGLSRED 154

                  2
148 AADMSSASNNFVFLKNEGLLDISFMLNACYDVVGEGIPFSPYICAGIGTDLVSMFEATNPKISYQGKLGLSYSISPEASV  227
155 A----IADKKYVVLKNEGITFMSLMVNTCYDITAEGVPFIPYACAGVGADLINVFKDFNLKFSYQGKIGISYPITPEVSA  230
                  6          7

                  4     ↓  *---3----------*
228 FIGGHFHKVIGNEFRDIPTIIPTGSTLAEKGNYPAIVILDVCHFGIELGGRFAF  281
231 FIGGYYHGVIGNNFNKIPVITPVVLEGAPQTT-SALVTIDTGYFGGEVGVRFTF  283
                  9          8
```

[1]Ehrlichia p28-OMP proteins 19 (top) vs. 14 (bottom). Peptides caged in pentamers (italicized) are identified by numbers above or below the peptide (See Figure 7). Arrow indicates a separation between two distinct caged peptides. Asterisks indicates possible immunogenic sites of conservation between p28-OMP 19 and 14.

Fig. 10.5 p28-OMP 19 and -OMP 14 peptides caged in pentamers. Ehrlichia p28-OMP proteins 19 (*top*) versus 14 (*bottom*). Peptides caged in pentamers (*italicized*) are identified by numbers above or below the peptide (See Fig. 10.6). *Arrow* indicates a separation between two distinct caged peptides. *Asterisks* indicate possible immunogenic sites of conservation between p28-OMP 19 and 14

E. chaffeensis organisms isolated from DH82 cells. The IGGHFHKV peptide-bound pentamer (#4 Table 10.1) consistently bound to the highest number of $CD19^{-}CD8^{+}$ T cells in all three experiments run (Fig. 10.6). A second carboxy-situated peptide, IGNEFRDI peptide-coupled pentamer (#3 Table 10.1) bound high numbers of $CD19^{-}CD8^{+}$ T cells in two experiments (Fig. 10.6). The EAHRYCAL peptide-coupled pentamer (#1 Table 10.1) bound $CD19^{-}CD8^{+}$ T cells in one experiment. Pentamer-coupled peptides IGGYYHGV (#9 Table 10.1) and IGNNFNKI (#8 Table 10.1) from p28-OMP 14 bound $CD19^{-}CD8^{+}$ T cells in one experiment (Fig. 10.6). Interestingly, IGGYYHGV (#9 Table 10.1) from p28-OMP 14 aligned with the IGGHFHKV p28-OMP 19 peptide (#4 Table 10.1) (Fig. 10.5). Similarly, IGNNFNKI (#8 Table 10.1) from p28-OMP 14 aligned with the IGNEFRDI (#3 Table 10.1) p28-OMP 19 peptide (Fig. 10.5). Therefore, there appear to be cross-reactive epitopes in p28-OMP 14 and p28-OMP 19 that induce $CD8^{+}$ T cells.

This review provides important information about the properties of the host response against *E. chaffeensis*. The absence of $CD4^{+}$ T cells causes some disruption in the ability of mice to clear the infection (Ganta et al. 2002, 2004), but neither $CD4^{+}$ T cells nor $CD8^{+}$ T cells are individually needed for the animals to cure the infection. We also discussed how the induction of effector memory responses in the mouse was highly efficient after both primary and secondary infections with *E. chaffeensis*. This is not surprising given that *E. chaffeensis* is a good T-cell immunogen and that effector memory cell mobilization is associated with protective immunity with *E. muris* (Thirumalapura et al. 2008).

Others have suggested that $CD8^{+}$ T cells might be more harmful than beneficial to the host during the immune response (Bitsaktsis et al. 2004; Feng and Walker 2004; Ismail et al. 2004; Bitsaktsis and Winslow 2006). Although these observations have been most frequently identified in animals infected with the more pathogenic IOE strain, there is some suggestion that this may also be true for *E. chaffeensis*, depending on the mouse strain (Feng and Walker 2004) and in humans (Dierberg and Dumler 2006; Pritt et al. 2011). Although *E. chaffeensis*-specific $CD8^{+}$ T cells are generated after experimental challenge, it does not appear that they are pathogenic. However, the presence of antigen-specific $CD8^{+}$ T cells after *E. chaffeensis* challenge suggests that $CD8^{+}$ T cells may play a role in the host immune system, even though they are not absolutely necessary for the host to cure the infection.

The predominant $CD8^{+}$ T-cell idiotype identified after an *E. chaffeensis* infection was bound by $H\text{-}2K^{b}$ pentamers presenting the IGGHFHKV peptide. The other two predominant $CD8^{+}$ T-cell idiotypes that have been identified after *E. chaffeensis* infection recognize SNYSFKYE and EAHRYCAL epitopes caged in pentamers. These idiotypes were generated by immunizing mice with whole organisms grown in DH82 macrophages. However, we found that T cells specific for p28-OMP 14 could also be generated. CD8 T cells were generated specific for peptides at positions equivalent to IGGHFHKV in p28-OMP 19 (peptide #4, Table 10.1) (See Fig. 10.5 for alignments) and IGNEFRDI in p28-OMP 19 (peptide #3, Table 10.1). These data suggest that T cells elicited by the bacteria grown in macrophages will induce T cells that can recognize P28 OMP 14; the major OMP expressed by the organisms grown in tick cells (Singu et al. 2005, 2006). There are some non-cross-

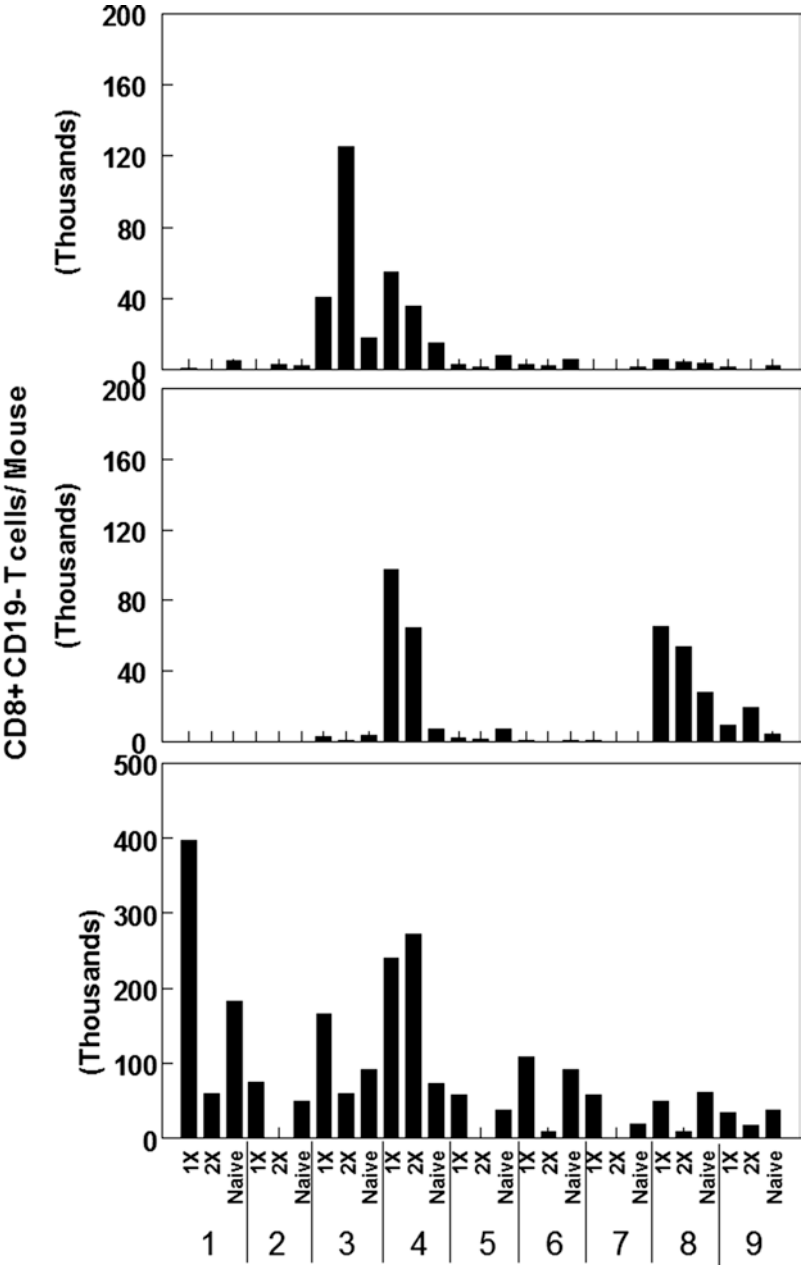

Fig. 10.6 Identification of *Ehrlichia chaffeensis* peptide-specific $CD8^{+}CD19^{-}$ T cells. H-$2K^{b}$-restricted MHC pentamers (peptide number indicated at the *bottom* of the graph) were used to determine *Ehrlichia chaffeensis*-specific $CD8^{+}CD19^{-}$ T cell numbers as described in the Materials and Methods. Mice experimentally challenged (*i.p.*) with *E. chaffeensis* grown in DH82 cells (1X, 2X, or naïve). *Top panel*, 51 and 67 days; *Middle panel*, 68 and 84 days; *Bottom panel*, 26 and 82 days. For each pentamer sample 5×10^{5} total cells were assessed, 2–3 mice were pooled per treatment group per experiment. Three independent experiments are shown

reactive epitopes such as EAHRYCAL (peptide #1, Table 10.1; AA positions 136–143, Fig. 10.5), as well.

Our work has expanded on the work of Nandi et al. (2007) to determine which *E. chaffeensis* $CD4^+$ T-cell epitopes were stimulatory. We used the RankPep algorithm to identify three p28-OMP 19 peptides that were capable of binding to IA^b which coincidentally also had varying amounts of overlap with *E. chaffeensis* peptides that were based on stimulatory IOE. These predicted peptides were also effective at inducing a proliferative response by T cells ex vivo; although only two of the three RankPep-predicted epitopes were as stimulatory as *Ehrlichia* peptides that were made based on IOE peptides. These data confirm the flexibility in the ability of MHCII to bind peptide (Brown et al. 1993) and the recognition of *Ehrlichia* antigen-specific helper T cells. We have not determined if the same T-cell idiotypes were stimulated by the overlapping peptides. What is of interest, however, is that the most stimulatory IA^b-binding peptide is a highly conserved sequence found in both p28-OMP 19 and p28-OMP 14 (Fig. 10.5, starting at AA107). There is 88 % homology between p28-OMP 19 and p28-OMP 14. Another possible $CD4^+$ T-cell epitope with 44 % amino acid homology (p28-OMP 19 aa238–252; Fig. 10.5) was not predicted by RankPep to be $CD4^+$ T-cell epitope. It is also important to point out that in outbred populations, the array of immunogenic peptides will be dependent on host MHC genotype. Therefore, it will be of future interest to determine if there are "hotspots" in p28-OMP 19 and p28-OMP 14 that serve as "universal immunogens".

1 Conclusions

There are novel *Ehrlichia* p28-OMP epitopes recognized by $CD4^+$ and $CD8^+$ T cells. There are also immunogenic p28-OMP 14 epitopes that have very high amino acid identity that cross-react with p28-OMP 19 epitopes and stimulate both $CD8^+$ T cells and $CD4^+$ T cells. Additional work will be needed to catalog the complete array of T-cell idiotypes that are generated during a successful host response to *E. chaffeensis*.

2 Detailed Methods Used to Study Host Immunity to *E. chaffeensis*

Mouse strains: Mice were originally obtained from the Jackson Laboratory (Bar Harbor, ME). C57BL/6J (B6 MHC-$II^{+/+}$ *Tlr4* $^{Lps\text{-}n}$) were used as wild-type controls. CD4 knock-out mice (CD4Δ; B6.129S2-$Cd4^{tm1Mak}$N8/J) have a targeted mutation to disrupt $CD4^+$ T-cell maturation leaving over 90 % of the circulating T cells as $CD8^+$ (Rahemtulla et al. 1991). CD8 knock-out mice (CD8Δ; B6.129S2-$Cd8^{tm1Mak}$N13/J) were deficient in functional cytotoxic T cells (Fung-Leung et al. 1991). Mice were

between 6 and 10 weeks of age at the beginning of the experiments. Mice were bred in the rodent facility of the Division of Biology at Kansas State University. Animal experiments were approved by the Institutional Animal Care and Use Committee.

In vitro cultivation of E. chaffeensis: The *E. chaffeensis* Arkansas isolate was cultivated in the canine macrophage cell line DH82 at 37 °C, 8 % CO_2 (Chen et al. 1995) or the ISE6 tick cell line (Munderloh et al. 1994) grown at 34 °C. Cultures with 80–90 % infectivity were used for experimental infection studies, as described by our group previously (Ganta et al. 2002, 2004, 2007).

Tissue collection and processing: Peritoneal exudate cells, spleen, and bone marrow were harvested from mice at various times post-infection as has been detailed previously (Ganta et al. 2002, 2004, 2007). Depending on the experiment, samples were used for biological assays, RNA analysis, and flow cytometry. Spleens were subdivided as follows: Part of the spleen was used to prepare RNA to quantitate bacterial load (see below). Spleens or splenic fragments were used for proliferation assays (see below). The tissue was homogenized, erythrocytes were lysed, and the cells were counted. One million spleen cells per well in 96-well plates were used in proliferation assays as have been described previously (Kopydlowski et al. 1992). A fraction of the spleen cells was also used for flow cytometric analysis (Ganta et al. 2004, 2007).

Bone marrow was washed from the femora and humerii and was used to determine the presence of bacteria. Peritoneal exudate cells containing predominantly macrophages were collected from *E. chaffeensis*-infected and uninfected control mice by washing the peritoneal lavage with 20 mL ice-cold, sterile PBS. These were also used for RNA isolation to determine the presence of bacteria.

RNA isolation: Total RNA from in vitro cultures, or cell pellet collected from 10 mL of peritoneal wash cells, or from about 50 mg of spleen were isolated by using a Tri-reagent RNA isolation kit as per the manufacturer's protocol (Sigma Chemical, St. Louis, MO). Final purified RNA pellets from peritoneal cells were resuspended in 50 μL of Tris-HCl buffer containing EDTA (TE buffer), and the pellets from liver and spleen tissue were resuspended in 100 μL of TE each. The purity and concentration of RNA was assessed using Nanodrop Spectrophotometer (Nanodrop Technologies, Wilmington, DE) by calculating the ratio between the optical densities at 260 and 280 nm. The absorbance ratio for all samples ranged between 1.8 and 2.0. The quality of RNA for a subset of samples was also confirmed by resolving them on a 1.5 % formaldehyde agarose gels. The RNA samples were also treated with RQ1 DNase (Promega, Madison, WI) for one hour at 37 °C to remove any trace amount of genomic DNA contamination before using for RT-PCR analysis.

Real-time quantitative RT-PCR: TaqMan-based real-time RT-PCR assay targeted to *E. chaffeensis* 16S rRNA gene was performed as described earlier (Sirigireddy and Ganta 2005). The amplification product formation was monitored in real-time by measuring the emitted fluorescence in the extension phase of the PCR cycles using the Smart Cycler system (Cepheid, Sunnyvale, CA). The reaction was regarded as positive for the presence of a template when amplified product formation resulted in

the detection of ten fluorescent units. The temperature cycle at which this occurred was regarded as the Ct value which was template concentration dependent (Sirigireddy and Ganta 2005).

Peptide synthesis and characterization: Peptides for $CD4^+$ T-cell stimulation were synthesized using solid-phase peptide synthesis (Applied Biosystems Model 431A peptide synthesizer; Foster City, CA) employing 9-fluorenylmethoxy-carbonyl (Fmoc) chemistries (Carpino and Han 1972; Fields and Noble 1990; Tomich et al. 1998). CLEAR amide resin (0.3 mmol/g; Peptides International, Louisville, KY) and N^{α}-Fmoc amino acids (Anaspec Inc., San Jose, CA) were used. All peptides were purified by HPLC (System Gold HPLC; Beckman Instruments, Inc., Fullerton, CA) using a reversed-phase C-18 column (Phenomenex, Torrance, CA) and eluted from the column using a linear gradient of 3.0 % min^{-1} of 10–90 % acetonitrile containing 0.1 % trifluoroacetic acid (TFA) at 1 mL/min. HPLC-purified peptides were characterized by matrix-assisted-laser desorption time-of-flight mass spectroscopy (MALDI-TOF/TOF; Bruker Ultraflex III, Bruker Daltronics, Billerica, MA). After characterization, peptides were lyophilized and stored as dry powders until used.

Proliferation assay. Spleens cells were collected from naïve mice (N), and *E. chaffeensis* inoculated (*i.p.)* mice previously challenged one or two times with 3×10^6 *E. chaffeensis* grown in DH82 cells. Lymphocytes were isolated from homogenized spleens and red blood cells were lysed by incubating in ammonium chloride lysis buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM Na_2EDTA, pH 7.3) for 5 min at 4 °C. Cells were centrifuged ($300 \times g$, 5 min) and washed two times in Dulbecco's Modified Minimal Essentials Medium (Hyclone Laboratories, Inc., Logan, UT) containing 1 % fetal bovine serum, 1 % Nu serum (BD, Bedford, MA), Glutamine plus (2 mM, Atlanta Biologicals, Atlanta, GA), 0.1 M HEPES and 10 % Opti-MEM (Invitrogen, Grand Island, NY) ($DMEM_2$). Spleen cells were suspended in $DMEM_2$ and were plated on 96-well plates (1×10^6 cells/well/100 μL) and were incubated at 8 % CO_2, at 37 °C for 5 days with the peptides described in Table 10.1 for a final peptide concentration of 0, 1, 10, or 25 μg/mL (final volume/well 200 μL). Experiments were done in triplicate. Proliferation was determined based on [^{3}H]-thymidine incorporation. [^{3}H]-Thymidine (24 μL/well; 0.025 μCi/μL) was added for the last 14 h of the incubation. Incorporation was determined with a scintillation counter (Microbeta Wallac Trilux. Perkin Elmer).

Stimulation Index was calculated as follows: $SI = [(\text{experimental/control}) \times 100]$, where controls cells were incubated with nonspecific peptide and experimental cells were incubated with *E. chaffeensis* peptides. A stimulation index of 100 reflects background/control proliferation.

Identification of Ehrlichia-specific $CD8^+$ T cells using pentamer staining: Mice were inoculated (*i.p.*) with *E. chaffeensis* one or two times. Mice received the first round injection at time 0 (first round, 1X) were rested for the indicated number of days and were then re-injected at the day indicated (second round, 2X); 51 and 67 days, 68 and 84 days and 26 and 82 days. Cells were collected on the last day indi-

cated. Naïve, unimmunized mice were used as normal controls. Three independent experiments were performed.

For class I ProVE pentamer staining, pentamers loaded with peptides were prepared by Proimmune (Sarasota, FL) and kept frozen at −20 °C until use. Pentamers were thawed and resuspended according to the manufacturer's specifications. Cells were also prepared using manufacturer's specifications. Briefly, isolated spleen cells were washed twice with wash buffer (0.1 % sodium azide, 0.1 % BSA in PBS) and resuspended at 2×10^6 cells/well in PBS. Cells were blocked with PBS-goat serum (50 %) for 30 min at 4 °C. Cell suspensions were then incubated with unlabelled pentamers for 10 min at room temperature. The cell suspensions were washed three times with wash buffer and centrifuged at 300 g for 5 min. Antibodies or isotype controls were added: allophycocyanin (APC)-conjugated fluorotag, PECy5-conjugated anti-CD19 or anti-IgG2b, and fluorescein isothiocyanate (FITC) anti-CD8 or anti-IgG2b. Samples were incubated for 20 min at 4 °C in the dark. Cell suspensions were fixed with Proimmune fix solution. For FACS analysis, cells were gated as live cells based on the forward scatter (FSC) and side scatter (SSC) parameters (Fig. 10.7, A and B panels). Gating of $CD8^+$ splenocytes was based on isotype controls (Fig. 10.7, panel C) to minimize nonspecific CD8 staining (Fig. 10.7, panel D). The number of pentamer binding cells was determined along with CD19-isotype (Fig. 10.7, panel E) and specific antibodies (Fig. 10.7, panel F). A total of 5×10^5 total cells were assessed from each sample. The % positive cells binding a pentamer (Fig. 10.7, bottom right panel, upper left quadrant) was multiplied by the total number of spleen cells collected per animal to estimate the number of peptide-specific T cells per mouse. Samples from 2 to 3 mice were pooled per treatment group per experiment.

Flow cytometry: Phenotypic analysis of spleen cells was performed by fluorescence-activated cell sorting as has been described previously by our group (Ortega et al. 2009; Potts et al. 2008). Briefly, 1×10^6 mouse spleen cells were aliquoted per well of round bottom, 96-well plates in a volume of 100 μL. Cells were pelleted and the supernatant was removed from the cells. Spleen cells were blocked with Hanks buffered salt solution (HBSS): goat serum (50:50; 50 μL) at 4 °C for 0.5 h. Fifty microliters of mouse-specific antibodies, anti-CD62L-APC (BD Pharmingen, Franklin Lakes, NJ), and anti-CD44-PE (eBiosciences, San Diego, CA) were added to the cell suspensions and incubated at 4 °C for 1 h. The cells were then washed twice in HBSS and resuspended in PBS containing 1 % formalin. Cells were analyzed with a FACSCalibur flow cytometer (Becton Dickson. Rockville, MD) and a minimum of 10,000 events were collected for each sample. Splenic lymphocytes were identified in forward-scatter versus side-scatter plots and assessed for CD44 and CD62L.

Statistics. Data are presented as the mean ± standard deviation of independent experiments. Numbers were analyzed by Student's *t* test (two-tailed, general) by using the StatMost Statistical Package (Data XIOM, Los Angeles, CA, USA). *P* values of <0.05 were considered significant.

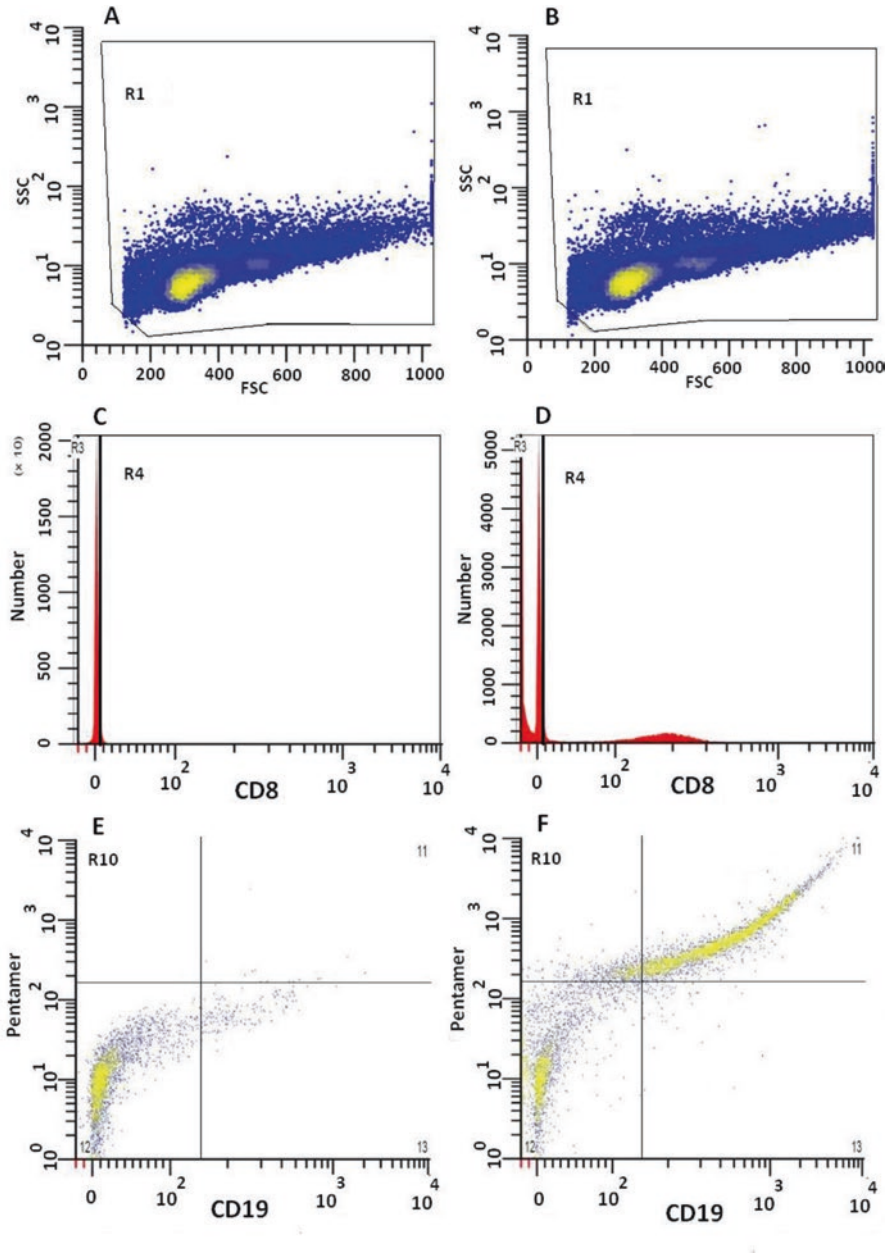

Fig. 10.7 Identification of $CD19^{-}CD8^{+}$ pentamer-binding T cells. T cells recognizing peptides caged in pentamers were gated based on forward- versus side-scatter to select live cells and omit debris (panels **A** and **B**). CD8-positive cells (panel **D**) were selected from the histogram output based on isotype antibody control staining (panel **C**). The number of pentamer binding cells was determined based on pentamer- (positively staining cells) and CD19 antibody- (negatively staining cells) specific staining (Region R10 in panel **F**. Panel **E** shows the isotype antibody controls staining)

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Chapter 11
Mutagenesis in *Ehrlichia* and *Anaplasma* Species: Its Application for Studies Focused on Understanding the Pathogenesis and Vaccine Development

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General Comments: Two families within the order *Rickettsiales* are *Anaplasmataceae* and *Rickettsiaceae* and contain many obligate intracellular Gram-negative bacteria (Dumler et al. 2001). The pathogens belonging to these two families are responsible for many important diseases in various vertebrate animals and people (Dumler et al. 2001). The *Anaplasmataceae* family has several recently identified intra-phagosomal pathogens of the genera *Anaplasma, Ehrlichia,* and *Neorickettsia* (Dumler et al. 2001). Bacteria belong to these three genera are very similar when visualized by microscopic examination after staining infected host cells following polychromatic staining, as they reside within a phagosome of an infected host cell. Cell tropism is a major distinguishable feature for identifying the organisms in a vertebrate host, while clinical signs share extensive similarity (McVey et al. 2013). *Ehrlichia* and *Anaplasma* species are harbored by ticks and are transmitted to vertebrate hosts during tick feeding, while *Neorickettsia* are fluke-borne pathogens. *Rickettsiaceae* family pathogens of the genus *Rickettsia* escape phagosomes and replicate within intracytoplasmic space and invade vascular endothelium (Dumler et al. 2001). Despite the enormous importance of the pathogens in causing diseases in people and various vertebrate hosts resulting in high morbidities and mortalities, studies on these pathogens are severely limited due to lack of genetic manipulation systems. Rickettsial pathogens are evolved to contain reduced genomes (Brayton et al. 2001; Sallstrom and Andersson 2005; Hotopp et al. 2006; Merhej and Raoult 2011), where majority of the genes of each pathogen may be essential for their survival in invertebrate and vertebrate hosts, thus making it a challenge to create mutations impacting the gene activity.

Anaplasmataceae family pathogens: *Anaplasmataceae* family within alphaproteobacteria has several species of tick-transmitted obligate, intracellular pathogens of the genera *Ehrlichia* and *Anaplasma*, which infect a wide range of vertebrate

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host species (Ristic and Huxsoll 1984; Rikihisa 1991; Dumler et al. 2001). In recent years, *Ehrlichia* and *Anaplasma* species are identified as causing potentially fatal human diseases (McDade 1990; Dumler and Bakken 1996; Schaffner and Standaert 1996). They include *E. chaffeensis*, the causative agent of human monocytic ehrlichiosis (HME) discovered in 1987 (Maeda et al. 1987; Dawson et al. 1991; Paddock et al. 1997), *A. phagocytophilum*, the human granulocytic anaplasmosis (HGA) agent that is reported in 1994 (Chen et al. 1994), *and E. ewingii*, the agent of human ewingii ehrlichiosis described in 1999 (Buller et al. 1999). *E. ewingii* is initially known as the canine pathogen responsible for granulocytic ehrlichiosis (Ewing et al. 1971; McDade 1990; Rikihisa 1991). In 2010, another *Ehrlichia*, namely *E. muris* like agent, is recognized as a new disease-causing agent in people (Pritt et al. 2011). *E. canis* is a canine monocytic ehrlichiosis agent (McDade 1990; Rikihisa 1991), which is also reported to cause infections in people in Venezuela (Perez et al. 1996, 2006). *E. ruminantium* is an economically important pathogen causing the disease, heartwater, in domestic and wild ruminants in sub-Saharan Africa and in parts of the Caribbean islands (Perreau et al. 1980; Uilenberg 1983; Kock et al. 1995; Perez et al. 1996). Infections in ruminant populations with *E. ruminantium* can reach up to 80 % mortality, particularly when the pathogen is introduced into a non-endemic area (Uilenberg 1983). Reports from South Africa suggest that *E. ruminantium* can also infect people (Allsopp et al. 2005; Louw et al. 2005). The Panola Mountain Ehrlichia (PME) is a pathogen highly homologous to *E. ruminantium* and infects goats and white-tailed deer in the USA (Loftis et al. 2006; Yabsley et al. 2008). PME infections are also documented in people (Reeves et al. 2008), although its potential to cause severe disease remains elusive. *A. phagocytophilum* is initially identified as the bovine and equine ehrlichiosis agent and later discovered to cause infections in people (Dumler et al. 1995, 2001). *A. marginale* is a bovine pathogen responsible for a major erythrocytic disease in cattle in the USA and many parts of the world (Brown and Barbet 2015).

Mutagenesis in Ehrlichia and Anaplasma species: Transposon mutagenesis is proven to be most valuable in creating random mutations within the genomes of *A. phagocytophilum, A. marginale,* and *E. chaffeensis* (Ristic and Huxsoll 1984; Felsheim et al. 2006, 2010; Chen et al. 2012; Cheng et al. 2013; Oliva Chavez et al. 2015). Targeted mutagenesis approach is also utilized in creating mutations within the genome of *E. chaffeensis* (Cheng et al. 2013). Although targeted mutations are reported in both *Anaplasma* and *Ehrlichia* species, they remain a challenge, possibly due to the essential nature of the genes selected for creating mutations.

Mutational analysis in Anaplasma species: Random mutagenesis is described for the first time in 2006 for *A. phagocytophilum* with the aid of the Himar1 transposase system (Felsheim et al. 2006). The authors produced insertion mutations at several genomic locations and the insertions included the expression cassette producing the green fluorescent reporter protein to facilitate monitoring the mutants by image analysis. Insertions within the genome of the pathogen are identified by rescue cloning and sequencing methods. This finding is the major milestone for mutagenesis experiments in *Anaplasma* and *Ehrlichia* species. Subsequently, this molecular tool is utilized to create additional mutations to assess the gene functions. For example, Chen et al. (2012) presented

evidence that a single transposon insertion into dihydrolipoamide dehydrogenase 1 gene of *A. phagocytophilum* is sufficient to impact infection-associated inflammation. In particular, the pathogen lacking the functional gene causes splenomegaly, increased splenic extramedullary hematopoiesis, and altered clinic-pathological abnormalities during mammalian colonization. The observed immunopathology also correlated with enhanced reactive oxygen species production and NF-κB signaling in macrophages.

Initial mutagenesis experiments in *A. marginale* with a Himar I transposon cassette plasmid containing the pathogen gene promoter, *tr*, resulted in the single homologous crossover event that is not mediated by the transposase system (Felsheim et al. 2010). This finding provides the first evidence suggesting that targeted mutations by homologous recombination are possible in *Anaplasma* species and possibly in *Ehrlichia* species. Genome-wide transcriptional profiling is carried out on this *A. marginale* mutant strain as it exhibited slow growing phenotype under in vitro culture conditions and in vivo in both cattle and ticks (Noh et al. 2011; Pierlé et al. 2013). The transcriptional analysis-identified individual genes such as fpba and rnhB are transcribed at significantly lower levels in the mutant compared to wild type and encode functions consistent with reduced bacterial growth (Pierlé et al. 2013). Significant transcriptional alterations are also observed in genes encoding for proteins involved in translation, translation elongation, and purine biosynthesis pathways. These data suggest that a relatively minor change in the genome can cause a pronounced phenotypic effect. Crosby et al. (2014, 2015) recently described similar random transposon mutagenesis approach for *A. marginale* where a single insertion mutant is generated with an insertion into a major immunogenic protein gene operon, Omp10. While the mutant has similar morphology and growth kinetics in vitro in both mammalian and tick cell cultures, it has an altered (reduced) infectivity and growth in cattle. Oliva Chavez et al. (2015) presented the first evidence demonstrating the requirement of a functional gene, the o-methyltransferase family 3 gene, for the replication of A. phagocytophilum in tick cells.

Mutational analysis in Ehrlichia species: Mutational analysis in *Ehrlichia* species is reported for the first time for *E. chaffeensis* by our research team (Cheng et al. 2013). Mutations described in this study included both the targeted and random mutagenesis. Targeted mutagenesis is a challenging task for obligate pathogens such as *E. chaffeensis* having reduced genomes, as majority of the genes are likely essential for the organism's life cycle in vertebrate and tick hosts. *E. chaffeensis* has a small genome of 1.18 megabase and contains 936 genes which includes genes encoding for 883 proteins (Hotopp et al. 2006). Our recent transcriptomic studies for this organism cultured in vitro in vertebrate macrophages and tick cells suggested that 94 % of the gene-based microarray representing 845 open reading frames are transcriptionally active in the organism (unpublished results). Consistent with this observation, Kuriakose et al. (2011) reported the expression of 80 % of all genes of *E. chaffeensis* during infection in human and tick cells. Assuming that the gene expression from the majority of genes is necessary for *E. chaffeensis* in support of its life cycle in vertebrate and tick hosts, identifying a genomic location within the organism for targeted mutagenesis can be a challenge. Other challenges in creating mutations are that *Ehrlichia* species cannot survive outside a host cell for a long period of time (Li and

Winslow 2003) and that the organisms do not harbor plasmids (Hotopp et al. 2006) and therefore the pathogens may not respond well to plasmid transformations.

Targeted mutagenesis: We investigated targeted mutagenesis extensively and the targets selected included six genomic locations assessed by homologous recombination and mobile group II intron-based methods (Cheng et al. 2013). We first evaluated various experimental conditions to identify protocols that work the best in transforming the organism with a plasmid and then various antibiotics are assessed for their usefulness in selecting the mutants. Subsequently, targeted mutagenesis is carried out by homologous recombination at a site, which appeared to be transcriptionally silent. The research is then expanded to six genomic locations using the modified mobile group II intron method, as recent studies demonstrate that it is an efficient method for creating targeted mutations in several Gram-positive and Gram-negative bacteria (Dumler and Bakken 1998; Whitlock et al. 2000; Paddock and Childs 2003; Demma et al. 2005). Independent of the methods used, targeted mutations are created; however, they survived in culture only for a short period of up to 8 days. We reasoned that the genomic regions selected for insertion mutations might represent regions that are necessary for the organism's persistent growth in culture. Alternatively, the antibiotic selection marker gene we chose (chloramphenicol acetyltransferase gene; CAT gene) to confer resistance against the antibiotic (chloramphenicol) or the antibiotic itself may have side effects impacting the growth of the mutants with resistant gene. We are now investigating the use of alternate antibiotic selection markers to develop more stable targeted mutations in *E. chaffeensis*. Indeed, recently we are able to identify a stable mutant by homologous recombination method when using a spectinomycin and streptomycin resistance gene in place of the CAT gene (Wang et al. unpublished results). This stable mutation is observed at a genomic region that has already been shown not to be critical for the pathogen's growth in vitro. More detailed investigations are now underway to expand the scope of targeted mutagenesis studies.

The choice of targeted mutagenesis methods (homologous or group II intron-based) also does not have an impact in mutant generation. We also learned that targeted mutations are possible only at certain genomic locations. For example, we could generate target mutations only at three out of six genomic targets evaluated (which included targets to disrupt the coding regions of Ech_0126, Ech_1136, and Ech_1143 and to create insertions within the intergenic regions located between the genes Ech_0039 and Ech_0040, Ech_0111 and Ech_0112, and Ech_0251 and Ech_0252). Independent of a mutagenesis method used, targeted mutations are achieved only at coding regions of genes Ech_0126 and Ech_1143 (gene encoding for an outer membrane protein, p28-Omp19). On the contrary, mutation at Ech_1136 gene (a gene homolog of Ech_1143 encoding for another related membrane protein, p28-Omp 14) is undetectable despite several attempts. Similarly, we could detect intergenic mutations only when we targeted to a noncoding region between Ech_0111 and Ech_0112 genes. Together, these data suggest that not all genomic regions are mutatable, possibly because of their critical requirement for the pathogen's continuous replication.

Random mutagenesis: Several independent experiments are performed to create mutations randomly within the genome of *E. chaffeensis* with the use of Himar

I transposon mutagenesis system (and our unpublished data). In vitro cultured *E. chaffeensis* propagated in ISE6 tick cells is more readily mutatable by random mutagenesis, although we are able to create mutations in *E. chaffeensis* when the organism is propagated in Vero cells (our unpublished results). We generated random mutations at numerous genomic locations of *E. chaffeensis*; we mapped 11 mutations and several more are under evaluation.

Mutations impacting the gene expression: Our recently characterized transposon-based mutants included insertions into the coding regions of three genes predicted to be expressing putative membrane proteins (genes Ech_0379, Ech_0601, and Ech_0660). The mutations caused the complete loss of gene expression, but without impacting growth of the organisms in vitro in either macrophage or tick cell cultures. The insertion mutation 18 base pairs downstream from the coding sequence of another gene, Ech_0230, also inhibited its mRNA synthesis. To assess if the insertion mutations located in non-coding regions have any impact on the genes surrounding the mutations, RNA expression from genes located immediately upstream and downstream to insertion sites for five clonally purified mutants is assessed (Cheng et al. 2015). Indeed, mutations in both non-coding and coding regions also have influence in altering the transcriptions from the immediate upstream and/or downstream genes. For example, the insertion mutation located at the 3′ end of the gene Ech_0202 causes a minor elevation of the transcription from Ech_0203 gene. Mutation downstream to the Ech_0284 gene increases the mRNA level from Ech_0285 gene. Similarly, mutation within the Ech_0379 gene causes decline in the mRNA from the Ech_0378 gene to undetectable levels similar to the loss of transcription from Ech_0379. The mutation downstream to the Ech_0479 causes an increase in mRNA produced from this gene and also activates the transcriptionally silent gene, Ech_0480. Intergenic mutation within the gene Ech_0660 not only causes transcriptional inactivation from itself, but also influences negatively in transcription to undetectable levels from the upstream gene, Ech_0659.

Mutations within the coding and/or non-coding regions of E. chaffeensis can cause attenuation in vertebrate hosts without impacting the acquisition by the tick vector: It is possible that the mutations at both intragenic and intergenic locations in *E. chaffeensis* may alter genome-wide expression changes and impair the organism's growth in vertebrate and tick hosts, as observed for *A. marginale* mutation (Pierlé et al. 2013). We discovered that insertion mutations at three intergenic locations and one intragenic location cause attenuation of the pathogen's growth in its reservoir host (white-tailed deer) and in an incidental host (dog), but appear to have no impact on the pathogen's continued survival in the tick host, *Amblyomma americanum* (Cheng et al. 2015).

The random mutants generated in *E. chaffeensis* are maintained well under in vitro culture conditions. Insertions within the coding regions of three putative membrane protein encoding genes causing the loss of their mRNA expression (genes Ech_0230, Ech_0379 and Ech_0660) also cleared rapidly from deer or dogs, while mutants with insertions at four non-coding regions, and one insertion within the coding region of Ech_0601 gene caused persistent infection similar to wild-type *E. chaffeensis* infection when assessed in the blood of infected animals for several

weeks (Cheng et al. 2013, 2015; Nair et al. 2014, 2015). A mutation in the non-coding region, with an insertion near Ech_0202 gene, is also undetectable in blood of infected deer or dogs. These data suggest that the mutations in certain non-coding regions also impact the bacterial survival in vertebrate hosts and likewise not all mutations within a coding region are detrimental to the organism. In summary, our in vivo infection studies demonstrated that disruptions within three genes encoding for putative membrane proteins of *E. chaffeensis* aided the hosts (deer and dogs) to rapidly clear the pathogen. Attenuation is also noted with an insertion at a noncoding region. These data suggest that the random mutagenesis approach is ideal in identifying genes essential for the persistent growth and pathogenesis of *Ehrlichia.*

We also studied if there are different gene requirements for the pathogen's acquisition and maintenance in ticks compared to vertebrate hosts by carrying out infection studies in *A. americanum* ticks with the mutants. We standardized a method of generating infected ticks by needle inoculation that is useful in assessing the infection status with mutant *Ehrlichia.* Independent of ticks receiving infections as a pool of mutants or as individual mutant, infections with the mutants persisted in ticks similar to the wild-type pathogen infection (Cheng et al. 2015). As the mutant organisms included those that are cleared rapidly from vertebrate hosts, the data suggest that pathogen gene requirements for the arthropod vector and vertebrate hosts are distinct.

1 Attenuated Mutants of *E. chaffeensis* Induce Protection Against Infection Challenge

Attenuated mutants confer protection against wild-type infection challenge in deer: The rapid clearance of the mutants in vertebrate hosts may have resulted due to the pathogen's inability to continuously overcome the host clearance. In particular, mutations at certain genomic locations leading to the loss of gene functions make the pathogen inefficient to sustain infection in the vertebrate hosts. We observed rapid clearance of the pathogen when mutations are present at four genomic locations; three mutations located within the coding regions of three different genes (Ech_0230, Ech_379, and Ech_0660) and one mutation in the noncoding region downstream to the coding region of Ech_0202 (Cheng et al. 2013, 2015). As these mutants survived only for few days prior to their complete clearance from a host, they are likely inducing sufficient host response valuable to protect against wild-type infection challenge. Indeed, our recent studies supported this hypothesis; two clonally purified attenuated mutants with insertions within the Ech_0379 and Ech_0660 genes offered protection against infection challenge (Nair et al. 2015; McGill et al. 2016). The protection against wild-type infection challenge is evident in both deer (the reservoir host) and dogs (an incidental host). Deer receiving infection with these mutants helped to rapidly clear wild-type pathogen infection when injected by intravenous (IV) route. In particular, all animals receiving prior vaccination with the attenuated mutants cleared the wild-type infection rapidly and the animals remained free of infection for over 7

weeks of assessment. Similarly, clonally purified mutants with insertions in Ech_0379 and Ech_0660 genes confer protective response in an incidental host, dog. Dogs initially vaccinated with Ech_0379 or Ech_0660 mutant and then challenged after a month with IV infection challenge with wild-type *E. chaffeensis* also resulted in the pathogen's rapid clearance from blood. The Ech_0660 mutant-infected group is tested positive only for the first week after infection challenge, while the Ech_0379 mutant group-challenged dogs are tested positive for the first 17 days post-challenge. The organisms are undetectable also in tissue samples assessed at the end point of the study in both deer and dogs. This is contrary to the animals receiving wild-type infection, which persists throughout the infection assessment in blood and in tissues at the terminal point of the study.

Attenuated mutants are also protective against tick-transmitted challenge: The value of attenuated mutants as vaccine in conferring protection against the natural route of infection challenge by tick transmission is also assessed. This experiment is carried out in dogs with the clonally purified Ech_0660 gene mutant (McGill et al. 2016). About 4 weeks after receiving the attenuated mutant vaccine, dogs are allowed to receive infection by tick transmission. Those dogs developed systemic infection less frequently compared to the ones that received only tick transmission of the pathogen and the wild-type infection is also undetectable in tissues in dogs vaccinated with the mutant while the wild-type infection persisted.

Together, above-described recent progress demonstrates that the random mutagenesis is a valuable tool in mapping the critical determinants of *Ehrlichia* and *Anaplasma* species and also greatly aids in the development of mutants suitable for conferring protection against the disease caused by the pathogens transmitted from infected ticks.

Future perspectives: Since the first description of the mutagenesis experiment in *A. phagocytophilum* a decade ago, the progress in this line of research is encouraging. Nonetheless, mutagenesis research is still at its infancy for the *Anaplasmataceae* pathogens. Research led by Ulrike Munderloh from the University of Minnesota paved the way for much of the advances with the transposon mutagenesis systems. Genomic integration by homologous recombination is described for both *Anaplasma* and *Ehrlichia* species. A single crossover event in *A. marginale* with a plasmid containing a segment of the pathogen DNA represents the first evidence of homologous recombination in *Anaplasma*. We reported targeted mutations at several genomic locations in *E. chaffeensis*. Despite these advances, establishing targeted mutagenesis protocols that work well and consistently in *Anaplasma* and *Ehrlichia* species require further refinement. The progress summarized here for tick-borne *Anaplasmataceae* family clearly demonstrates the importance of both random and targeted mutagenesis methods. The methods are most valuable in understanding the pathogenic mechanisms of the organisms, defining the critical genomic determinants of the pathogens for tick and vertebrate host infections, and in developing attenuated vaccines against the diseases. Future advances in mutagenesis will likely aid in furthering our understanding of the molecular basis for vector and host associations, host cell tropism, evasion mechanism, determining the pathogenesis, and in continuing efforts in developing novel methods of control.

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Chapter 12
Epidemiology, Molecular Biology, and Pathogenic Mechanisms of *Ehrlichia* Infections

Xue-jie Yu and David H. Walker

1 Introduction

Ehrlichia are tick-borne, obligately intracellular Gram negative bacteria. The diseases caused by *Ehrlichia* are called ehrlichioses, which are zoonotic and are transmitted through tick bite. Ehrlichioses have been recognized as veterinary diseases much earlier than as human diseases. *Ehrlichia canis* is the first bacterium that was named as *Ehrlichia* in 1935 (Donatien and Lestoquard 1935), but the first *Ehrlichia* organism *Ehrlichia ruminantium* was discovered in 1925. At the time, it was named *Rickettsia ruminantium* (Allsopp 2010). Human ehrlichiosis caused by *E. chaffeensis* was discovered in 1980s (Maeda et al. 1987). Since then, other human ehrlichioses have been discovered including ewingii ehrlichosis and *E. muris* ehrlichiosis. The recognized *Ehrlichia* species include *E. canis*, *E. chaffeensis*, *E. muris*, *E. ewingii*, and *E. ruminantium*. All these organisms cause animal and human infections.

2 Definition and Phylogeny of *Ehrlichia*

Despite more than a century of research on *Ehrlichia* and in this era of molecular biology, the definition of *Ehrlichia* is not well-delineated. New species of *Ehrlichia* are continually designated, even though there are no criteria for a new species.

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Table 12.1 Percentage similarity of sequences of 16S rRNA gene of recognized *Ehrlichi*a species

		1	2	3	4	5	6	7	8	9	10	11
1.	*E. chaffeensis* AF416764	***	99.9	99.1	99.1	98.2	98.1	98.2	97.9	98.9	97.2	97.5
2.	*E. chaffeensis* U60476		***	99.2	99.2	98.2	98.2	98.2	97.9	98.9	97.3	97.6
3.	*E. muris* CP006917			***	100	98.3	98.2	98.3	98.6	98.8	97.8	97.9
4.	*E. muris* EMU15527				***	98.3	98.2	98.3	98.6	98.8	97.8	97.9
5.	*E. canis* EF011110					***	99.9	100	97.6	98.5	97.1	97.3
6.	*E. canis* AF162860						***	99.9	97.5	98.4	97.1	97.2
7.	*E. ovina* AF318946							***	97.6	98.5	97.1	97.3
8.	*E. ewingii* EEU96436								***	99.8	97	97.1
9.	*E. ewingii* NR_044747									***	98.2	98.3
10.	*E. ruminantium* NR_074513										***	99.7
11.	*E. ruminantium* X62432											***

Currently, *Ehrlichia* are classified using their 16S rRNA gene (*rrs*) sequence homology. Genetic analysis of the organisms in the genus *Ehrlichia* indicates that different strains of the same species have 99–100 % homology in the 16S rRNA gene sequence. Comparison of the sequence of 16S rRNA gene of the recognized species of *Ehrlichia* indicates that the closest homology among *Ehrlichia* species except for *E. canis* and *E. ovina* is 99.1 % between *E. chaffeensis* and *E. muris* (Table 12.1), which can be a criterion for classification of *Ehrlichia* organism. The sequence homology between *E. canis* and *E. ovina* is 99.9–100 %, indicating that these two organisms should be a single species, i.e., *E. ovina* is a strain of *E. canis* because *E. canis* was discovered much earlier than *E. ovina. E. canis* and *Candidatus* Ehrlichia regneryi are phylogenetically in the same cluster (Fig. 12.1) and share 99 % *rrs* DNA sequence homology (Table 12.1).

When more strains of *Ehrlichia* are added for comparison, the species boundaries become less distinct (Table 12.2). For example, *Ehrlichia* sp. HF (DQ647318) from an *Ixodes ricinus* tick in France is 99.2–100 % homologous to *E. muris*, and 99 % homologous to *E. chaffeensis* by *rrs* homology (Table 12.2). In this case, we think DNA sequence homology and phylogeny should be considered together. A genogroup contains *Ehrlichia* sp. HF (DQ647318) and several uncultured *Ehrlichia* species that are phylogenetically closely related to *E. muris*.

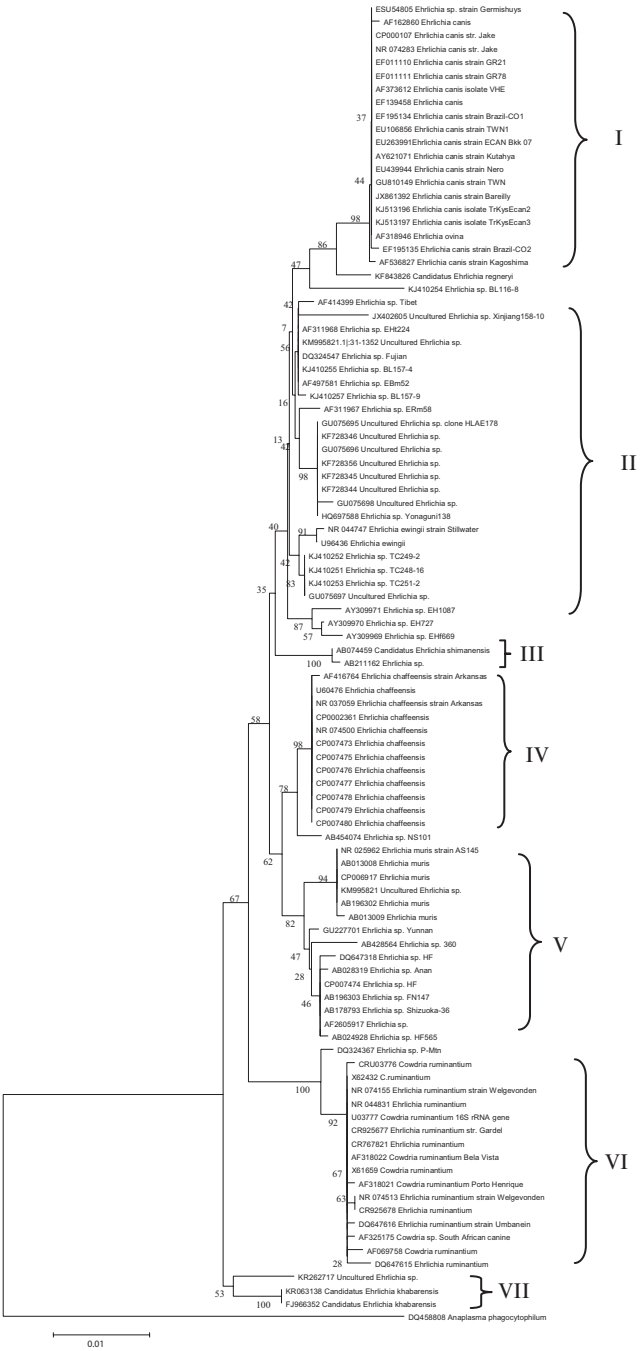

Fig. 12.1 Phylogenetic analysis of *Ehrlichia* species uisng the sequences of 16S rRNA gene. *Ehrlichia* species are classified into 7 geneotypes/species.

Table 12.2 Percent similarity of sequences of 16S rRNA gene of recognized *Ehrlichia* species

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1. AB196302 *E. muris*	***	98.6	98.6	98.5	98.7	99.2	98	97.7	97.9	97.2	97.1	97.2	97.2	97.5	97.1	96.8	97.1	97.9	97.8	97.6
2. AB024928 *Ehrlichia* sp.		***	98.8	99.8	99.9	99.8	98.6	98.3	98.3	97.7	97.5	97.6	97.6	97.8	97.2	96.9	97	98.3	98.3	97.8
3. AB428564 *Ehrlichia* sp.			***	98.8	98.9	99.2	98.2	97.9	97.9	97.2	97.3	97.3	97.6	97.8	96.9	97	96.8	97.9	97.9	97.8
4 AB028319 *Ehrlichia* sp.				***	99.9	99.8	98.5	98.3	98.3	97.7	97.5	97.5	97.5	97.7	97.1	96.9	96.9	98.2	98.3	97.7
5. AB178793 *Ehrlichia* sp.					***	99.8	98.7	98.4	98.4	97.8	97.6	97.7	97.7	97.8	97.3	97	97.1	98.4	98.4	97.9
6. DQ647318 *Ehrlichia* sp.						***	99	98.7	98.8	98.1	98	98.1	98.2	98.5	97.7	97.7	98	98.8	98.6	98.6
7. NR074500 *E. chaffeensis*							***	99.6	99	98.3	98.3	98.4	98.4	98	97.7	97.1	97.5	98.2	98.4	98.3
8. AF147752 *E. chaffeensis*								***	99.1	98.5	98.5	98.5	98.5	98.1	97.3	97.4	97.3	98.6	98.7	98.2
9. AF497581 *Ehrlichia* sp.									***	99.3	98.8	98.9	98.8	98.6	97.8	97.9	97.8	99.3	99.1	98.9
10. JX402605 *Ehrlichia* sp.										***	98.1	98.2	98.1	98	97.1	97.2	97.1	98.7	98.5	98.3
11. EF195135 *E. canis*											***	99.9	99	98.2	97.5	97.4	96.8	98.5	98.6	97.8
12. AF318946 *E. ovina*												***	99.1	98.1	97.4	97.4	96.9	98.5	98.5	97.9
13. KF843826 *E. regneryi*													***	98.2	97.2	97.3	97	98.2	98.5	97.9
14. U96436 *E. ewingii*														***	97.4	97.4	96.7	98.3	98.4	98.3
15. NR044831 *E. ruminantium*															***	96.2	97.1	97.7	98	97.6
16. KJ410254 *Ehrlichia* sp.																***	96.6	97.4	97.6	97.4
17. KR262717 *Ehrlichia* sp.																	***	97.2	97.4	97.5
18. KF728346 *Ehrlichia* sp.																		***	99	98.6
19. AY309970 *Ehrlichia* sp.																			***	98.6
20. AB074459 *E. shimanensis*																				***

Ehrlichia organisms can be classified into 7 genotypes/species (Fig. 12.1). Each species/genotype contains core species and satellite species. The first genotype is *E. canis* and *E. ovina* as core species (99.5–100 % *rrs* identity) and *Candidatus Ehrlichia regneryi* as satellite species (99 % *rrs* identity). Both *E. ovina* and *Candidatus Ehrlichia regneryi* may be strains of *E. canis*. The second genotype has *E. ewingii* as species and many uncultured *Ehrlichia* from ticks and animals as satellite species. These satellite species are mainly from Asia, especially from China. The classification of these species needs to be determined. The third genotype is *Candidatus Ehrlichia shimanensis* from ticks collected in Japan, which is most closely related to the uncultured *Ehrlichia* from China in the *E. ewingii* group (98.4–99.1 % *rrs* homology, Table 12.2). The fourth genotype is *E. chaffeensis*. The fifth genotype is *E. muris*, which can be further divided into two subtypes. The sixth genotype is *E. ruminantium*. The seventh genotype is *Candidatus* Ehrlichia khabarensis from ticks in the Russian Far East.

3 Biphasic Life Cycle Inside Host Cells

Electron microscopy showed that *E. chaffeensis* are polymorphic bacteria (0.2–2.0 μm in diameter), but mainly consists of two morphologic forms: a small dense-core cell (DC) and a large reticulate cell (RC) (Popov et al. 1995). A biphasic developmental cycle has been demonstrated. In the biphasic life cycle, the small DC is infectious, binds to, and is internalized into host cells, and then develops into a larger replicating RC inside a membrane-lined compartment that resembles late endosomes. After replication in expanding inclusions, the mature RCs transform into DCs and are released from the host cells (Zhang et al. 2007). DCs are more resistant to oxidative stress than RCs (Cheng et al. 2011).

4 Molecular Biology

4.1 Genome Reduction

Inside the host cell, *Ehrlichia* are evolved to obtain nutritional components from the host cell rather than expend energy to synthesize them. For a long period of intracellular life, *Ehrlichia* genes involved in metabolism have mutated, lost functions that are not required to synthesize molecules that are available from the host cell, and were eventually deleted. Therefore, *Ehrlichia* evolved a small genome through genome reduction process. The size of the *Ehrlichia* genome (approximately 1.2 MKbs) is only a quarter of the size of the genome of a free living bacterium *Escherichia coli*. All organisms in *Rickettsiales* including *Ehrlichia* have a diminished ability to synthesize amino acids compared to their closest free-living relatives (Yu et al. 2009). However, unlike members of the Rickettsiaceae family, *Ehrlichia* and *Anaplasma* are capable of producing all major vitamins, cofactors, and nucleotides, which could confer a beneficial role in the invertebrate vector or the vertebrate host (Dunning Hotopp et al. 2006).

4.2 Tandem Repeat and Ankyrin Repeat Proteins

The *Ehrlichia* genome encodes several surface proteins that are involved in host–pathogen interactions, including tandem repeat proteins and ankyrin repeat containing proteins (Dunning Hotopp et al. 2006; Mavromatis et al. 2006). The major immunodominant proteins of *E. chaffeensis* contain acidic serine-rich tandem repeats, including P120 (Yu et al. 1997), TRP47, and TRP32 (Doyle et al. 2006; Luo et al. 2008, 2009). *Ehrlichia chaffeensis* P120 and TRP47 are associated with dense-core ehrlichiae, and P120 has been demonstrated to be an adhesin (Popov et al. 2000).

Ankyrin (Andrić) repeat, one of the most widely existing protein motifs in nature, appearing as repeat units in *Ehrlichia* proteins, consists of 30–34 amino acid residues and exclusively functions to mediate protein–protein interactions, involved in a multitude of host processes including cytoskeletal motility, tumor suppression, and transcriptional regulation (Li et al. 2006; Mosavi et al. 2004). Ank consists of two anti-parallel α-helices connected to the next repeat unit via a loop region (Mosavi et al. 2004). Ank is very common in eukaryotes, but *ank* genes encoding heterogeneous Ank proteins are present in facultative or obligate intracellular bacteria, including *Ehrlichia* and *Anaplasma. Ehrlichia chaffeensis* Ank protein is a 200 kDa protein (Ank200), which is translocated to the nuclei of *Ehrlichia*-infected mononuclear phagocytes where it interacts with an adenine-rich motif in promoter and intronic *alu* elements (Zhu et al. 2009). The association of Ank200 with *alu* elements suggests that Ank200 could affect gene transcription globally through *alu*-mediated transcriptional control mechanisms. The global analysis of binding sites of Ank200 demonstrated that this protein binds to multiple regions distributed on nearly every chromosome via direct DNA interaction or with other DNA-binding proteins (Wakeel et al. 2010). *Ehrlichia chaffeensis* Ank200 interacts with apoptosis, ATPase, and transcriptional regulatory genes, and genes associated with pathogenesis and immune evasion including TNF-α, Jak2, and CD48 (Lee and Rikihisa 1996).

5 Epidemiology and Clinical Manifestations of Ehrlichioses

5.1 E. canis *Infection*

Ehrlichia canis was discovered in Algeria in 1935. The first case in the United States was reported in 1963. It was not until about 1968–1970, during the Vietnam war, when the full pathologic potential of *E. canis* was first recognized. A severe epizootic episode of ehrlichiosis occurred among U.S. military dogs resulting in hundreds of cases of morbidity and mortality.

Ehrlichia canis is transmitted through the bite of the brown dog tick *Rhipicephalus sanguineus*. However, *E. canis* is not transmitted transovarially in *R. sanguineus* (Groves et al. 1975). *Ehrlichia canis* organisms can infect and multiply in the midgut and salivary gland, but not in the ovary of ticks (Smith et al. 1976).

Adult brown dog ticks efficiently transmitted *E. canis* to susceptible dogs for 155 days after detachment as engorged nymphs from a dog in the acute phase of ehrlichiosis. Adult ticks that had similarly engorged on a dog in the chronic phase of ehrlichiosis failed to transmit *E. canis* to susceptible dogs, suggesting that acutely infected dogs are more important than chronically infected dogs in transmission of *E. canis* (Lewis et al. 1977). The brown dog tick is the most widespread tick, is more commonly found in warmer climates, and is associated with human habitations and domestic dogs in urban, suburban, and rural environments. The brown dog tick can be found in most populated areas in the United States and is rarely associated with uninhabited wild or forested areas (Faherty and Maurelli 2008). The brown dog ticks feed on a variety of hosts, but domestic dogs are the preferred host in the United States (Dantas-Torres 2010). *Rhipicephalus sanguineus* is unique among tick species as it can complete its entire life cycle indoors; therefore, infestations in homes or kennels can become established rapidly (Lord 2001). *Dermacentor variabilis* has been experimentally demonstrated to successfully transmit *E. canis* to dogs after transstadial ehrlichial passage after molting (Johnson et al. 1998).

Ehrlichia canis primarily infects dogs and occasionally infects humans and felines (Bowman et al. 2009). Antibody to *E. canis* has been detected in 64 of 250 patients suspected to have vector-borne diseases in Montenegro (Andrić 2014). Human infection with *E. canis* has been occasionally reported in South America. Two studies reported asymptomatic infection by *E. canis* in humans in Venezuela and Mexico (Perez et al. 1996). A third study reported that six human patients with clinical signs compatible with human monocytic ehrlichiosis and admitted to the emergency clinic in Lara State, Venezuela, were identified as infected by detection of *E. canis* 16S rRNA by gene-specific polymerase chain reaction (PCR) (Perez et al. 2006). These studies showed that *E. canis* can cause asymptomatic to severe disease in humans. This was the first report of *E. canis* infection of human patients with clinical signs of HME. Compared with the U.S. strains, 16S rRNA gene sequences from all six patients had the same base mutation as the sequence of the *E. canis* Venezuelan human *Ehrlichia* (VHE) strain previously isolated from an asymptomatic human.

Clinical manifestations of E. canis: In dogs experimentally infected with *E. canis*, incubation periods ranged between 17 and 22 days (mean = 19 days). Clinical signs typical of ehrlichiosis included mucopurulent ocular discharge, lymphadenopathy and malaise with accompanying pyrexia, leukopenia, and thrombocytopenia. Pyrexia, thrombocytopenia, erythrophagocytosis, and vacuolation of the cytoplasm of monocytic cells were observed 1–4 days prior to detection of morulae. *Ehrlichia canis* morulae in peripheral blood lymphocytes can be detected at 30 days post-exposure.

5.2 Human Monocytic Ehrlichiosis

In April 1986, a medical intern scanning the peripheral blood smear of a presumed spotless Rocky Mountain spotted fever patient discovered morulae inside monocytes of the patient (Paddock and Childs 2003; Fishbein et al. 1987).

These inclusions resembled morulae of *E. canis* previously known in the United States solely as veterinary pathogens (Maeda et al. 1987; Paddock and Childs 2003). The pathogenic bacterium formally named *E. chaffeensis* was used in cell culture in 1991 (Anderson et al. 1991; Dawson et al. 1991). The disease caused by *E. chaffeensis* was named human monocytic ehrlichiosis because the major target cells of *E. chaffeensis* are monocytes. Since then, epidemiologic studies indicated that human monocytic ehrlichiosis is a widespread and a significant public health problem in the United States. Human monocytic ehrlichiosis became a reportable disease in 1999 (Centers for Disease Control and Prevention). The number of ehrlichiosis cases due to *E. chaffeensis* reported to CDC has increased steadily since the disease became reportable; from 200 cases in 2000 to 961 cases in 2008, but the annual case fatality rate has declined (Centers for Disease Control and Prevention). The lone star tick, *Amblyomma americanum,* is considered the vector of *E. chaffeensis* (Anderson et al. 1993; Ewing et al. 1995). Ehrlichiosis is most frequently reported from the southeastern and south-central United States, from the eastern seaboard extending westward to Texas. The areas from which cases are reported correspond with the known geographic distribution of the lone star tick. These states include Oklahoma, Missouri, and Arkansas, which reported 30 % of *E. chaffeensis* infections (Paddock and Childs 2003).

5.3 Human Ewingii Ehrlichiosis

Ehrlichia ewingii causes granulocytic ehrlichiosis in dogs and was discovered to cause human infection in 1999. *Ehrlichia* DNA detected in leukocytes from four patients from Missouri by a broad-range *rrs* PCR assay matched that of *E. ewingii*, but not *E. chaffeensis* (Buller et al. 1999). *Ehrlichia ewingii* morulae were identified in neutrophils (Buller et al. 1999). *Ehrlichia ewingii* is also transmitted by the lone star tick in the US (Anziani et al. 1990).

The clinical manifestations of *E. ewingii* infection in humans include fever, headache, and thrombocytopenia, with or without leukopenia. Although previous literature suggests *E. ewingii* primarily affects those who are immunocompromised (Buller et al. 1999) or infected with HIV (Paddock et al. 2001), a recent study showed that most cases occurred among immunocompetent patients; among 55 cases between 2008 and 2012, only 26 % were those for whom immune status were immunosuppressed (Heitman et al. 2016). *Ehrlichia ewingii* infections are impossible to distinguish from *E. chaffeensis* infections based on clinical signs alone, and some proportion of cases currently reported as *E. chaffeensis* infection may actually be due to *E. ewingii*. There is no currently available serologic test that can distinguish these agents, and surveillance for *E. ewingii* is currently based on detection of the organism through molecular-based diagnostic tests. A total of 55 cases of *E. ewingii* were reported to CDC from 2008 to 2012 (Centers for Disease Control and Prevention) and none was reported to be fatal.

5.4 E. muris *Infection in Humans*

Ehrlichia muris was first described from the vole *Eothenomys kageus* as well as from murid rodents in Japan (Kawahara et al. 1993; Wen et al. 1995) and *E. muris*-like (EML) organism was described to infect humans in the US in 2009 (Pritt et al. 2011). Since its initial identification, at least 69 persons exposed in Wisconsin and Minnesota have been confirmed to have been infected with the EML organism (Johnson et al. 2015). EML infection in humans causes fever, malaise, headache, lymphopenia, thrombocytopenia, and elevated liver-enzyme levels. All recovered after receiving doxycycline treatment. EML organism has been detected from the deer tick *Ixodes scapularis* collected in Minnesota and Wisconsin (Pritt et al. 2011; Telford et al. 2011).

6 Pathogenic Mechanisms

The pathogenesis of severe ehrlichial infections is mediated principally by the immune response. Immunocompetent patients who die with HME have few ehrlichiae in their tissues, suggesting host-mediated pathogenic mechanisms. In contrast, severely immunocompromised patients with HME such as those with human immunodeficiency virus-acquired immunodeficiency syndrome have overwhelming *E. chaffeensis* bacterial loads, suggesting that the organisms themselves also can damage the host directly. Extensive studies in mice experimentally infected with highly virulent *Ixodes ovatus* ehrlichia (IOE) and *E. muris*, which causes persistent subclinical infection, have elucidated some unique immunopathologic mechanisms. IOE induces a pathogenic CD8 T cell response with tissue damaging overproduction of TNF-α and suppression of protective immunity by overproduction of IL-10 by CD4 T cells and FoxP3 T regulatory cells (Ismail et al. 2004, 2006, 2007). These immune responses to *Ehrlichia* infection are mediated by type I interferon (IFN-1), inflammasome activation, an early neutrophil response, NK cells, production of numerous chemokines and chemokine receptors, and Nod-2 activation. IFN-1 activation appears to lead to noncanonical inflammasome activation, decreased autophagic ehrlichial clearance, and immunosuppressive decreased IFN-γ/IL-10 ratio with fewer protective CD4 T cells and NKT cells, which are important sources of anti-ehrlichial IFN-γ (Yang et al. 2015; Zhang et al. 2014). The early neutrophil response results in an increased bacterial load and immunopathology associated with increased chemokines that may be the mechanism of increased NK cells and CD8 T cells in the lesions (Yang et al. 2013). Nod-2 activation results in decreased protection and increased bacterial load associated with increased CD8 T cells and decreased NKT cells, CD4 T cells, and IFN-γ. IL-18 and IL-1β derived from inflammasome activation play roles in mediating the decreased protective immunity and occurrence of severe immunopathology (Chattoraj et al. 2013).

7 Manipulating Host Immune System

Ehrlichia are obligate intracellular Gram negative bacteria, which invade and multiply inside host phagocytes including macrophages, monocytes, and neutrophils, and for some organisms, endothelial cells. Phagocytes are the result of highly evolved host defenses to destroy invading pathogens. However, *Ehrlichia* organisms have evolved multiple strategies to subvert host innate immune responses to create an environment that is suitable for the organisms to reside and multiply inside the host cells.

7.1 Invading the Host Cell

Ehrlichia has a special ability to enter and replicate inside host cells (macrophages/monocytes, neutrophils, and endothelium). The P120 surface protein of *E. chaffeensis* is able to mediate recombinant *E. coli* to enter Vero cells and is expressed only on the infectious dense-core cell of *E. chaffeensis*, but not on the non-infectious reticulate form of *E. chaffeensis* (Popov et al. 2000). In a recent study, another *E. chaffeensis* outer-surface protein, EtpE, was shown to bind to a specific host cell-surface protein, DNase X, and this ligand-receptor interaction is required to induce the entry of *E. chaffeensis* into its host cells. Mice immunized with the recombinant EtpE protein are resistant to *E. chaffeensis* challenge. Mice lacking DNase X are resistant to *E. chaffeensis* infection (Kumar et al. 2013).

7.2 Evading Lysosomal Destruction

Once inside the cytoplasm, the pathogen prevents *Ehrlichia*-containing vacuole (ECV) fusion with lysosomes, an essential condition for *Ehrlichia* to survive inside phagocytes, but the mechanism of inhibiting the fusion of the phagosome with lysosomes is unclear. *Ehrlichia chaffeensis*-containing vacuoles contain the late endosomal marker Rab7 and are acidified at approximately pH 5.2, suggesting that the *E. chaffeensis* vacuole is an acidified late endosomal compartment. *Ehrlichia chaffeensis* inhibits phagosome-lysosome fusion by modifying its vacuolar membrane composition, rather than by regulating the expression of host genes involved in trafficking (Cheng et al. 2014).

7.3 Evading Host Cell Pattern Recognition Receptors

Pattern recognition receptors (PRRs) are a primitive part of the innate immune responses because they evolved before other innate immune system components and adaptive immunity were evolutionarily developed. Toll-like receptors (TLRs)

are a type of PRR that recognize pathogen-associated molecular patterns (PAMPs). There are a total of 10 human and 12 mouse TLRs named from TLR1 to TLR13 and each recognizes and is activated by a particular pathogen molecule. TLR2 recognizes Gram-positive bacterial lipoproteins, and peptidoglycan activates TLR2 in conjunction with either TLR1 or TLR6; lipopolysaccharide (LPS) is detected by TLR4; flagellin is detected by TLR5; poly I:C, a double-stranded RNA (dsRNA) analog, is detected by TLR3; unmethylated DNA and CpG-oligodeoxynucleotides (CpG-DNA) are detected by TLR9; and single-stranded RNA and its synthetic analogs resiquimod, imiquimod, and loxoribine activate TLR7 (Takeda et al. 2003; Takahashi 2008).

In the evolution of its intracellular life cycle, *Ehrlichia* organisms have deleted genes encoding cell wall components that strongly stimulate PRRs including LPS and peptidoglycan. Due to the lack of these cell wall components, *Ehrlichia* organisms are not recognized by PRRs and do not stimulate proinflamatory cytokines via these components.

7.4 Inhibiting Host Cell Apoptosis

Apoptosis is a process of programmed cell death that occurs in multicellular organisms (Green 2011). Apoptosis is characterized by DNA fragmentation, chromatin condensation, cytoplasmic shrinkage, and cell death without lysis or damage to neighboring cells. Bacteria, viruses, and parasites can either induce or prevent apoptosis to augment infection. Many bacterial pathogens that cause apoptosis target immune cells such as macrophages and neutrophils because these cells would otherwise kill the pathogens (Faherty and Maurelli 2008). In contrast, because they multiply inside host cells, *Ehrlichia* have evolved a mechanism to inhibit apoptosis of infected host cells in order to prolong the opportunity for intracellular pathogen growth that requires host cell survival. *Ehrlichia chaffeensis* and *E. muris* inhibit apoptosis through up-regulating NF-kB (nuclear factor kappa-light-chain-enhancer of activated B cells) (Mathema et al. 2013; Zhang et al. 2004). NF-kB is found in almost all animal cell types and is involved in cellular stress responses such as that caused by bacterial or viral infection, cytokine stimulation, free radicals, ultraviolet irradiation, and oxidized low-density lipoprotein stimulation (Gilmore 2006). NF-kB controls DNA transcription, cytokine production, and cell survival and plays a key role in regulating the immune response to infection. When the cell is not stimulated, the NF-kB dimers are sequestered in the cytoplasm by inhibitors of kB (IkBs). IkB contains multiple copies of ankyrin sequence repeats, which mask the nuclear localization signals (NLS) of NF-kB proteins to sequester NF-kB in the inactive state in the cytoplasm (Jacobs and Harrison 1998). In *Ehrlichia muris*-infected cells, IkB is degraded resulting in activation of NF-kB. NF-kB complex is then freed to enter the nucleus where it can "turn on" the expression of specific genes that have DNA-binding sites for NF-kB nearby. The activation of these genes by NF-kB then leads to a cell survival response, or cellular proliferation.

Ehrlichia ewingii infects neutrophils. Neutrophils generally have a short life span and naturally undergo apoptosis within 6–12 h after release into the peripheral blood from the bone marrow (Akgul et al. 2001), which is even shorter than the time for *Ehrlichia* to replicate and mature into dense core cells (Zhang et al. 2007). To overcome the short life of the neutrophil, *E. ewingii* infection delays canine neutrophil spontaneous apoptosis by maintaining the mitochondrial membrane potential in neutrophils (Xiong et al. 2008).

7.5 Dampening the Host Cell Immune Response

Both cellular and humoral immunity are important in elimination of *Ehrlichia* infection. Resistance of mice to sublethal challenge of *Ixodes ovatus* ehrlichia (IOE) is CD4-, but not CD8-, dependent and requires the IL-12p40-dependent cytokines, IFN-γ, and TNF-α, but not IL-4. In response to IOE antigens, CD4 T cells purified from infected mice proliferate in vitro and produce IFN-γ, which can rescue IFN-γ-deficient mice from fatal infection (Bitsaktsis et al. 2004). Wild-type, C57BL/6J mice are resistant to *E. chaffeensis*, but major histocompatibility complex class II (MHCII) knockout mice lacking helper T cells develop prolonged infections,$CD4^+$ T-cell-deficient mice clear the infection, but the clearance requires 2 weeks longer than in wild-type mice. These data suggest that the cell-mediated immunity orchestrated by $CD4^+$ T cells is critical for conferring efficient clearance of *Ehrlichia* (Ganta et al. 2004).

Although major roles are clearly played by T cells, antibodies can also control *Ehrlichia* infection in both normal and immunocompromised SCID mice and can protect the latter from lethal infection. Much of the humoral immune response is directed at the bacterial outer membrane proteins (OMPs). The antibodies (mostly IgG2a) can mediate bacterial clearance from tissues as early as 24 h after administration and require host Fc receptors for their function(s). One possible mechanism is that antibodies or immune complexes trigger microbicidal activities in infected macrophages that lead to the elimination of bacteria residing inside host macrophages. Alternatively, it is proposed that antibodies opsonize bacteria exposed during intercellular transfer. This notion is supported by studies that have demonstrated the presence of bacteria in the extracellular milieu during infection and suggests that our understanding of the behavior of the bacterium in the host may be key to our understanding of its susceptibility to antibody-mediated host defenses (Winslow et al. 2003). Survival of mice infected with highly virulent *Ixodes ovatus* ehrlichia requires both CD4 and CD8 T cells as well as antibodies (Ismail et al. 2004).

To survive inside animal hosts, *Ehrlichia* down-regulates host immune responses. *Ehrlichia chaffeensis* infection does not stimulate host cell cytokines that activate innate and adaptive immunity to intracellular bacteria. *Ehrlichia chaffeensis* does not stimulate IL-12, IL-15, and IL-18 production (Zhang et al. 2004). These cytokines play fundamental roles in stimulating NK cells and T helper 1 cells to produce gamma interferon (IFN-γ), which then activates macrophages to kill phagocytosed bacteria. IL-12 and IL-15 also activate NK cells and cytotoxic T lymphocytes to kill cells infected with intracellular bacteria. Thus, deficient production of IL-12, IL-15, and IL-18 may help *E. chaffeensis* to evade host innate and adaptive immunity.

Another intracellular bacterium, *Mycobacterium tuberculosis* (Nau et al. 2002), the intracellular protozoan *Leishmania major* (Carrera et al. 1996), and fungus *Histoplama capsulatum* (Marth and Kelsall 1997) inhibit IL-12 production. Thus, intracellular pathogens may have convergently evolved the ability to survive inside the macrophage by repressing IL-12 production.

MHC class II receptors are found on the surface of antigen presenting cells (B cells, macrophages, dendritic cells), but may be expressed by other cells (e.g., epithelial and endothelial) within inflammatory lesions (Day 2011). MHC class II molecules, first identified as antigen-presenting elements, interact with CD4 inflammatory (Th1) and helper (Th2) T-cells and are recognized as signal transduction molecules that regulate macrophage function (Day 2011). The expression of the MHC class II molecules is also necessary for CD4 T cell maturation. *Ehrlichia canis* infection downregulates MHC class II receptor expression in DH82 cells, suggesting a possible mechanism by which *E. canis* evades the immune system (Harrus et al. 2003).

Ehrlichia chaffeensis can survive by inhibiting critical signaling in monocyte activation pathways linked to pattern recognition receptors. *Ehrlichia chaffeensis* infection downregulates the expression of several pattern recognition receptors, including CD14, TLR2, TLR4, and transcription factor PU.1. *Ehrlichia chaffeensis* inhibits the activation of ERK 1/2 and p38 MAPK by LPS treatment in monocytes, suggesting that the inhibition of p38 MAPK by *E. chaffeensis* is involved in the suppression of several downstream signaling pathways (Harrus et al. 2003).

8 Conclusion

Ehrlichia are tick-borne, Gram negative intracellular bacteria, including *E. chaffeensis*, *E. canis*, *E. muris*, *E. ewingii*, and *E. ruminantium*. Human infections have been reported by all the *Ehrlichia* species. Human ehrlichioses have been mainly reported in the United States and have not yet reported in Asia, despite the existence of *Ehrlichia* in the area. Due to their obligately intracellular lifestyle, *Ehrlichia* reside in a stable environment with plenty of nutrients; therefore, *Ehrlichia* can afford to mutate and delete genes involved in cell wall components such as LPS and peptidoglycan and genes involved in metabolism, which resulted in a small genome. Without typical bacterial cell wall components, *Ehrlichia* become stealthy to the host cell innate immune system that recognizes pathogen-associated molecules. In addition to a stealthy cell wall, *Ehrlichia* have also evolved other strategies to subvert the host innate immune system such as inhibition of apoptosis and inhibition of fusion of *Ehrlichia*-containing vacuole with lysosome.

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Chapter 13
Ehrlichia ruminantium: The Causal Agent of Heartwater

Isabel Marcelino, Philippe Holzmuller, Frédéric Stachurski, Valérie Rodrigues, and Nathalie Vachiéry

1 Introduction

The *Rickettsiales Ehrlichia ruminantium* causes heartwater, an infectious, non-contagious tropical disease of ruminants. Heartwater (also called cowdriosis) is one of the most important tick-borne diseases of livestock in Africa (Vachiéry et al. 2014;), being a notifiable disease listed by the World Organization for Animal Health (Allsopp 2015).

Heartwater occurs wherever the *Amblyomma* spp. ticks are present, i.e. in nearly all the sub-Saharan countries of Africa and in the surrounding islands Madagascar, La Réunion, Mauritius, Zanzibar, the Comoros Islands, The Cap Vert and São Tomé. The disease is also reported in the Caribbean (Guadeloupe and Antigua), from where it threatens the American mainland (Vachiery et al. 2008a; Molia et al. 2008; Kasari et al. 2010; Vachiéry et al. 2013). It belongs to the 12th most important animal transboundary diseases listed by the US Homeland Security department for American mainland (Roth et al. 2013).

All domestic and wild ruminants can be infected, but the former appears to be the most susceptible. Non-indigenous ruminants that are moved into affected areas are particularly sensitive to heartwater, and mortality rates up to 90 % are observed. In enzootic areas, indigenous cattle, less infested by the vector ticks than introduced animals, have developed resistance to heartwater (Minjauw and McLeod 2003). For instance, in heartwater enzootic areas in southern Africa, it is estimated that mortalities due to the disease are more than double to those due to

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other tick borne diseases such as bovine babesiosis (mostly caused by *Babesia bovis* and *B. bigemina*) and bovine anaplasmosis (caused by the *Rickettsiales Anaplasma marginale*). In 2005, human and canine cases potentially due to *E. ruminantium* were reported in South Africa (Allsopp and Allsopp 2001; Allsopp et al. 2005b), and since then *E. ruminantium* infection has been considered as a potential zoonosis (Chitanga et al. 2014) although any other human case has been reported until now.

The average natural incubation period is 2–3 weeks, but it can vary from 10 days to 1 month. In most cases, heartwater is an acute febrile disease, with a sudden rise in body temperature. The most common macroscopic lesions are hydropericardium, hydrothorax and pulmonary oedema, giving the name "heartwater" to the disease. A clinical diagnosis of the disease is based on the presence of *Amblyomma* spp. ticks on the animals or in the environment, nervous signs, and presence of transudates in the pericardium and thorax on post-mortem examination. Nowadays, efficient molecular diagnostics are available for a reliable diagnosis.

Heartwater control can be achieved using several strategies such as vector chemical control, treatment of animals, chemoprophylaxis and vaccination. Currently, four vaccine strategies against heartwater have been developed: the "infection and treatment method" using live bacteria followed by antibiotherapy, immunization by infecting animals with in vitro attenuated bacteria (Jongejan et al. 1993; Jongejan 1991; Faburay et al. 2007; Zweygarth et al. 2005), immunization with inactivated in vitro grown bacteria (Martinez et al. 1994; Vachiery et al. 2006; Maass and Dalhoff 1995; Marcelino et al. 2007, 2015a) and recombinant vaccines (Simbi et al. 2006; Sebatjane et al. 2010; Pretorius et al. 2002). Nevertheless, the problems caused by high genetic and phenotypic diversity shown in restricted areas still remain, hampering the development of a fully effective vaccine for widespread application (Allsopp and Allsopp 2007; Barbet et al. 2009). Moreover, the knowledge of immune response modulation during heartwater is still limited.

To develop improved therapeutics, it is thus fundamental to increase knowledge on *E. ruminantium* biology and pathogenesis. The complete genome sequences of three *E. ruminantium* strains are currently available (Frutos et al. 2006b; Collins et al. 2005), but at the moment there is no method available for the genetic manipulation of this bacterium and little is currently known on whether specific genes are actually expressed in living organisms. For this reason, additional research on heartwater disease is essential. Currently, global and integrative high-throughput approaches such as functional genomics including transcriptomics and proteomics are being used to increase the knowledge on *E. ruminantium* biology in the frame of bacteria–host–vector interactions.

This book chapter aims at providing a state-of-the-art in the epidemiology, development of vaccine and immunology of heartwater and to give updated insights in the biology of the bacterium *E. ruminantium*.

2 The Etiologic Agent of Heartwater: *Ehrlichia ruminantium*

2.1 Classification

The causal organism of heartwater is an obligate intracellular bacterium, previously known as *Cowdria ruminantium* (Cowdry 1925, 1926). In 2001, Dumler and co-workers defined after *16S ribosomal DNA* and *groESL heat shock operon* genes comparisons that all members of the tribes *Ehrlichieae* and *Wolbachieae* had to be transferred to the family *Anaplasmataceae* and that the family *Rickettsiaceae* had to be eliminated (Dumler et al. 2001). Molecular evidence led to reclassification of several organisms in the order *Rickettsiales*, the causal agent of heartwater being now classified as *Ehrlichia ruminantium* (Dumler et al. 2001). In 2013, the order *Rickettsiales* was reorganized through 16S and 23S gene comparisons and now it includes the families *Rickettsiaceae*, *Anaplasmataceae* and *Midichloriaceae* (Ferla et al. 2013). The *Anaplasmataceae* family still includes the four genera *Ehrlichia*, *Anaplasma*, *Wolbachia* and *Neorickettsia*. The genus *Ehrlichia* includes *E. ruminantium*, *E. chaffeensis, E. canis* and *E. muris* while the genus *Anaplasma* contains *Anaplasma bovis* (formerly *E. bovis*), *A. marginale*, *A. centrale, A. platys* (formerly *E. platys*) and *A. phagocytophilum* (formerly *E. phagocytophila*), and the genus *Neorickettsia* includes now the formerly named *E. sennetsu* and *E. risticii* (Dunning Hotopp et al. 2006; Dumler et al. 2001; Rikihisa 2010). The genus *Wolbachia* includes *Wolbachia pipientis* (Fig. 13.1).

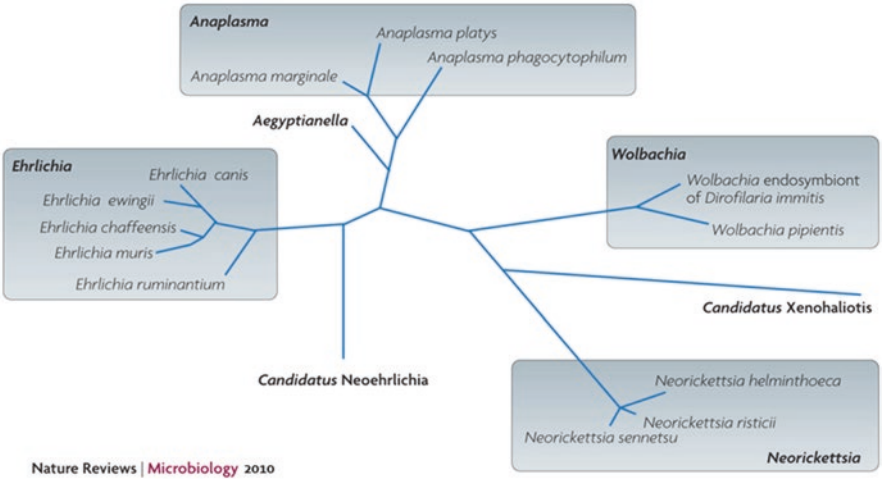

Fig. 13.1 Phylogram of the Family *Anaplasmataceae*. This phylogram is constructed based on 16S rRNA sequences of these species. Family *Anaplasmataceae* contains four genera: *Ehrlichia, Anaplasma, Neorickettsia*, and *Wolbachia* according to Dumler and co-workers (Dumler et al. 2001) and as reviewed by Rikihisa (2010))

2.2 Ehrlichia ruminantium *Colonies Morphology In Vivo and In Vitro*

Histopathological examination of brain smears reveals variable numbers of *E. ruminantium* colonies discernible in the cytoplasm of capillary endothelial cells after Giemsa staining (Fig. 13.2A); they can also be observed in lung smears (Van de Pypekamp and Prozesky 1987; Prozesky 1987b; Van Amstel et al. 1987).

A detailed characterization of *E. ruminantium* morphology in mammalian host cells was possible in 1985, when the first in vitro cultivation of the organism in a calf endothelial cell line was described (Bezuidenhout et al. 1985). Electron microscopy reveals two morphologically distinct forms: one that develops within membrane-bound vacuoles reticulate bodies (RB or reticulate cells, RC) forming colonies called morula, and that differentiate into the free infectious forms (elementary bodies, EB) (Jongejan et al. 1991). Morula are arranged in grapefruit and close to the nucleus inside endothelial cells; they are dark purple when coloured with Giemsa whereas elementary bodies are bright pink outside lysed cells (Fig. 13.2B).

Currently, the organism is propagated in vitro most reliably not only in endothelial cells (from cattle, sheep, goats, wild African mammals (Smith et al. 1998), human (Totte et al. 1993) and murine origins) but also in primary neutrophil cultures and macrophage cell lines (Yunker 1995). Several studies also showed that *E. ruminantium* is able to infect tick cell lines (Bell-Sakyi et al. 2000; Bell-Sakyi 2004) as well as cells from non-endothelial origin such as Chinese Hamster Ovary cells (CHO) and Baby Hamster Kidney Cells (BHK) (Zweygarth and Josemans 2001, 2003). These cultures are generally performed in small tissue culture systems but *E. ruminantium* can also be cultured in stirred tanks using microcarriers for large-scale production (Totte et al. 1993; Pedregal et al. 2008; Marcelino et al. 2006). *E. ruminantium* can also be propagated experimentally by inoculating infected blood from reacting animals or infected tick homogenate into a susceptible animal. Blood from the infected animal is then collected during hyperthermia and incubated in vitro on endothelial cells. *E. ruminantium* isolation from blood can take several weeks and several media exchanges must be performed to stimulate bacterial growth (Marcelino et al. 2005, 2006).

3 *Ehrlichia ruminantium* Biology

3.1 Ehrlichia ruminantium *Tick Transmission*

Heartwater has been transmitted experimentally by 12 species of *Amblyomma* ticks: *A. variegatum, A. hebraeum*, *A. pomposum*, *A. gemma*, *A. lepidum*, *A. tholloni*, *A. sparsum*, *A. astrion*, *A. cohaerens*, and *A. marmoreum*, *A. maculatum* and *A. cajennense* (Bezuidenhout and Bigalke 1987). *A. variegatum* is the most important heartwater vector with a worldwide distribution, followed by *A. hebraeum* which is only present in southern Africa. Five species (*A. tholloni*, *A. sparsum*, *A. gemma*,

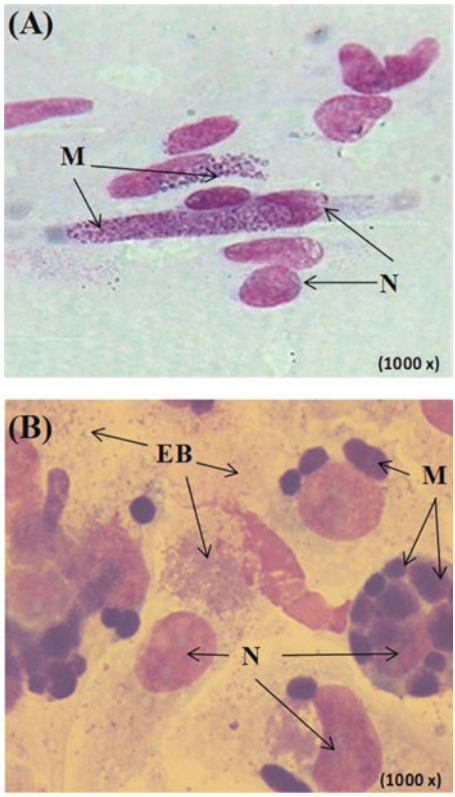

Fig. 13.2 *Ehrlichia ruminantium* colonies morphology (**A**) in vivo (brain smear from heartwater-infected goat stained with Giemsa showing numerous colonies of *E. ruminantium*) and (**B**) in vitro (endothelial cell monolayer stained with RAL 555 showing morula and infectious extracellular bacterium, after host cell lysis). *N* stands for nucleus of endothelial cells, *M* for morula (*E.ruminantium* colonies inside the host cell containing the intracellular form of the bacterium, the reticulate bodies) and *EB* for extracellular infectious *E. ruminantium* elementary bodies. (CIRAD photos: Nathalie Vachiéry and Isabel Marcelino)

A. cohaerens and *A. marmoreum*) have not been implicated in field outbreaks either because they are confined to forest areas or because of their host preference (Martinez 1997). The presence of *A. maculatum* and *A. cajennense* in the Americas threatens the American mainland from the introduction of the disease (Vachiery et al. 2013). In the Caribbean, *A. variegatum* is also known as the Senegalese tick (Barre et al. 1995) and the Antigua gold tick (Pegram et al. 2004).

Amblyomma spp. ticks are three-host ticks (one host *per* developmental stage and moulting or egg laying on the ground after engorgement) (Fig. 13.3). There is no trans-ovary transmission of *E. ruminantium*, however there is transstadial transmission. *Amblyomma* ticks become infected during the larval and/or nymphal stages when they feed on heartwater-infected domestic and wild ruminants (Martinez 1997). *Amblyomma* spp. are vividly coloured and decorated ticks, especially the males (Fig. 13.4). Different stages of *A. variegatum* ticks are shown in Fig. 13.4. In tropical regions, the life cycle of *Amblyomma* ticks shows important seasonal variations: adults infest their hosts during the rainy season, with a peak at the beginning of the season; larvae infest their hosts at the end of this rainy season and disappear rapidly from the environment, because of high sensitivity to desiccation, as soon as the rains cease; and nymphs attach to their hosts mainly at the beginning of the dry season. Heartwater is therefore a seasonal disease in these areas, observed mainly at the beginning of the rainy and dry seasons. In regions with equatorial climate, i.e. where the dry

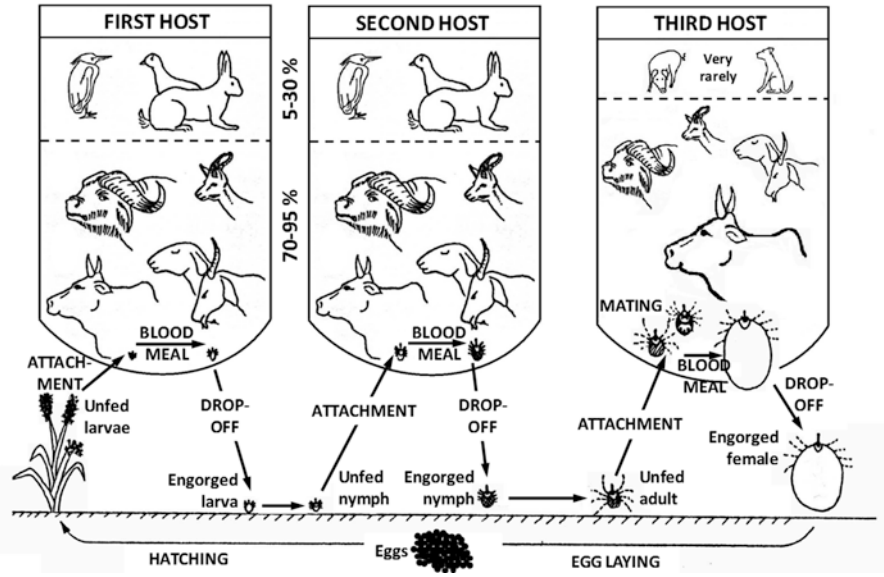

Fig. 13.3 Life cycle of a typical three-host tick (such as *Amblyomma variegatum*) feeding on three separate individual cows (by Frédéric Stachurski)

season is short or nonexistent, nymphs and adults may infest their hosts, and therefore transmit heartwater, all the year round. Such a situation is observed in French West Indies, but also in the humid and coastal areas of African countries and Madagascar.

The minimum period required for transmission of the parasite after tick attachment is between 27 and 38 h in nymphs and 21–75 h in adults (Bezuidenhout 1988), the pathogen being generally transmitted only after 3–4 days of attachment. Almost 100 % larvae or nymphs feeding on a clinically affected ruminant can acquire infection, which does not seem to lead to mortality or reduced survival of the ticks. After natural recovery or treatment, ruminants can become reservoir of *E. ruminantium* for months but the presence of the bacterium in the blood is not permanent; only part of the infesting ticks can pick up the bacterium from these animals.

Unfed nymphs sampled in the environment have an infection rate of 3 % whereas that of unfed adults is 8–20 %, according to different studies and regions (Mahan et al. 1998b). This allows the regular infection of cows and, therefore, the maintenance of enzootic stability when tick control is not drastically implemented. It has actually been demonstrated that regular infection of cows allows the vertical transmission of *E. ruminantium* to calves in utero (Deem et al. 1996). Calves can thus acquire early infection either by their dam, either by rapid infection by nymph or adults ticks, for those born during the adequate infestation periods. These infections, occurring when calves are protected by passive immunity provided by colostrum, enable early development of active immunity and persistence of enzootic stability.

On the contrary, infestation level of small ruminants by adult ticks is too low to enable systematic acquisition of immune protection by lambs and goat kids born during the rainy season. As nymphs infest their hosts only during a few months, at

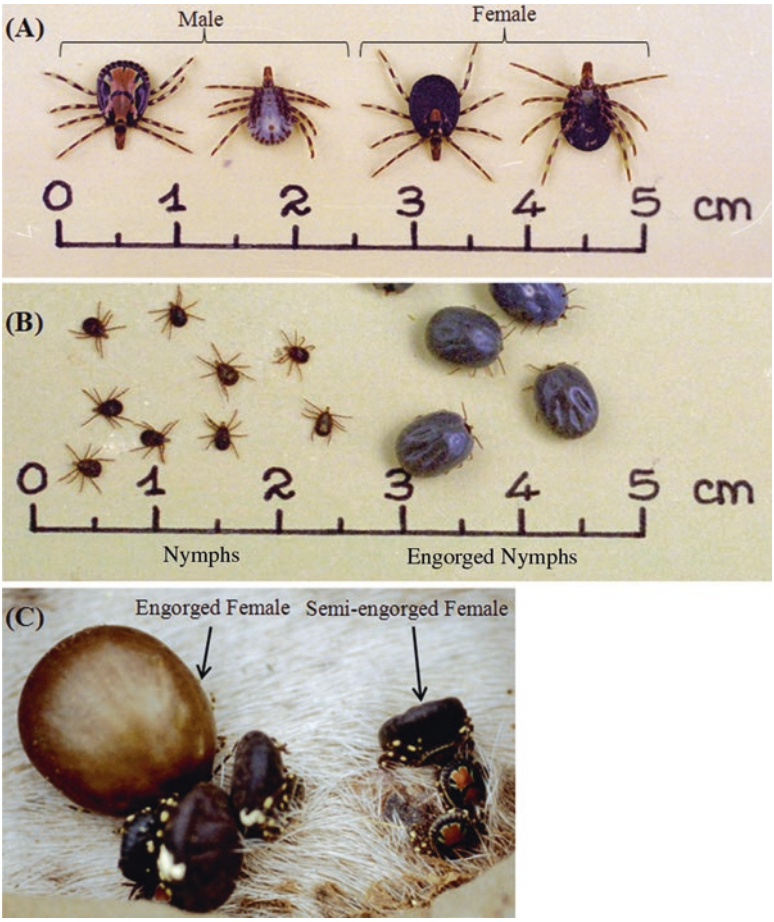

Fig. 13.4 The vector *Amblyomma variegatum*. (**A**) Male (dorsal and ventral positions) and female ticks (dorsal and ventral positions), (**B**) unengorged and engorged nymphs, (**C**) male and female on animals (CIRAD pictures: Frédéric Stachurski). The ruler aims at showing the large difference in size between *A. variegatum* nymphs and adults

the beginning of the dry season, in tropical areas, small ruminants born at other periods do not have the occasion to acquire active immune protection. They thus have no enzootic stability, which explain why local goats and sheep suffer mortalities due to heartwater, unlike local cattle breeds reared under traditional systems.

3.2 The Developmental Cycle of E. ruminantium

As above mentioned, *E. ruminantium* has a biphasic developmental cycle in host mammalian cells with two morphologically distinct forms, the extracellular infectious elementary body (EB) and the intracellular metabolically active reticulate

body (RB) (Fig. 13.2B). EBs are small (0.2–0.5 μm in diameter) and, after cell colonization, they reside within intracytoplasmic inclusions where they convert into the larger (0.75–2.5 μm) non-infectious RBs (Prozesky 1987a; Marcelino et al. 2005). The RBs multiply by binary fission, rapidly filling the inclusion (named morula), which expands in size (Prozesky 1987a). RBs re-condense back into EBs towards the end of the cycle and are then released from the host cell (Figs. 13.5 and 13.6). Microscopic observation of in vitro-cultivated *E. ruminantium* demonstrated the

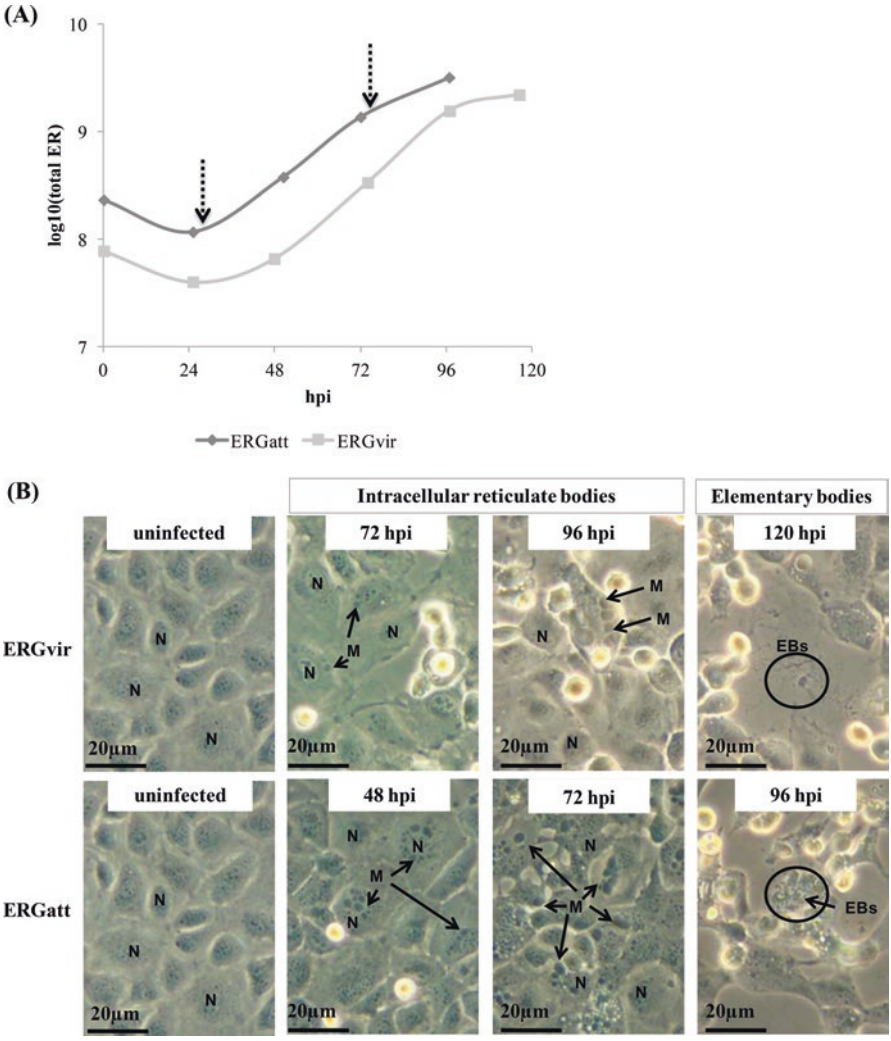

Fig. 13.5 Representative growth kinetics of *E. ruminantium* Gardel virulent (ERGvir) and attenuated (ERGatt) strains obtained by (**A**) real-time PCR targeting *map-1* gene (*dashed arrows* represent the time of total medium exchange) and (**B**) reverse phase microscopy (N stands for host cell nucleus, M for morula and EBs for elementary bodies) (Marcelino et al. 2015b)

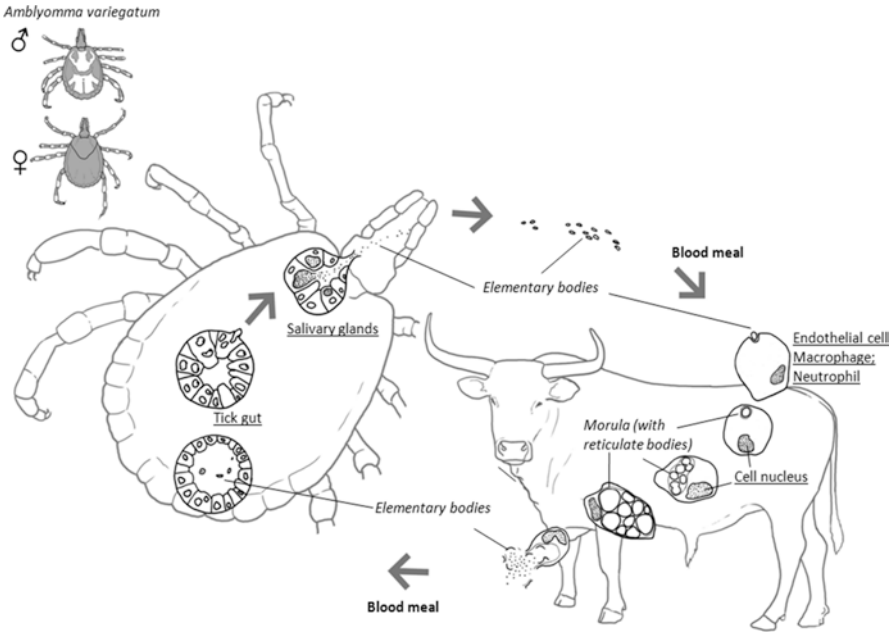

Fig. 13.6 *Ehrlichia ruminantium* life cycle (Marcelino et al., 2012b)

presence of intracellular RBs 2–4 days after infection (Marcelino et al. 2005, 2015b) (Fig. 13.5). A high number of EBs are observed after rupture of endothelial cells 5–6 days after infection (Jongejan et al. 1991; Moumene and Meyer 2015b; Marcelino et al. 2005) (Fig. 13.5). The relation between the stage of development and time post-infection depends on the strain and its adaptation to in vitro conditions. For instance, the virulent *E. ruminantium* Gardel strain (isolated in Guadeloupe, FWI) lyses 5 days post-infection whereas the attenuated Gardel phenotype lyses after 4 days (Marcelino et al. 2015b) (Fig. 13.5). In culture, EBs lose their infectivity within a few hours (Marcelino et al. 2005), but the organism, together with suitable cryoprotectants, may be viably preserved in liquid nitrogen for years (Marcelino et al. 2007; Brayton et al. 2003; Vachiery et al. 2006).

E. ruminantium developmental cycle and its infectivity within the tick are poorly understood. Transmission of the bacterium from the host to the vector occurs during an infected blood meal; *E. ruminantium,* present in the blood of the ruminant, initially develops in the gut epithelial cells of the attached tick and subsequently invades the salivary glands (Fig. 13.6). After attachment of the next tick stage to a new host, *E. ruminantium* develops and multiplies during few days in the salivary glands before being injected to the host via saliva during the blood meal. A single infected nymph or adult is able to cause the disease because of high multiplication of *E. ruminantium* in acini of salivary glands during the first phase of tick blood meal. More generally, tick saliva is thought to play a major role in the transmission of the bacterium from the vector to the vertebrate. Indeed, as other

ixodid ticks, *Amblyomma* spp. secrete a cocktail of immunomodulatory molecules in their saliva during blood-feeding that help to control the activity of host immunocompetent cells and, as a consequence, favour the establishment of *E. ruminantium* in the host target cells. One well described process is the neutralization of host cellular communication through the binding of specific saliva molecules to cytokines that have important roles in innate and adaptive immunity. A pioneering study focused on modulation of interleukin (IL)-8 that plays a critical role in inflammatory processes, and demonstrated that tick salivary gland molecules are able to bind to IL-8 preventing binding of the chemokine to its specific receptor (Kocakova et al. 2003). Further analyses demonstrated that tick saliva targets different cytokines providing a gateway for tick-borne pathogens that helps explain why ticks are such efficient and effective disease vectors. In particular, anti-IL-2, IL-4, IL-8 (CXCL8), MCP-1 (CCL2), MIP-1 (CCL3), RANTES (CCL5) and eotaxin (CCL11) activities were evidenced in *A. variegatum* salivary gland extracts (Hajnicka et al. 2005; Vancova et al. 2007, 2010a; Peterkova et al. 2008). Interestingly, the level of anticytokine activity depends on the species, developmental stage (adult or nymph), as well as on the number of days the tick has been feeding (Vancova et al. 2010b). The local immunodepression induced by tick saliva will probably indirectly help the initial multiplication of the bacteria, which probably takes place in reticulo-endothelial cells and macrophages in the lymph nodes draining the tick biting site. From here, the bacteria are disseminated via the blood stream to invade endothelial cells of blood vessels of various organs where further multiplication occurs (Du Plessis 1970).

4 Epidemiology

4.1 Heartwater Geographical Distribution

Heartwater is present in sub-Saharan Africa, the Comoros islands, including Mayotte, Madagascar, and the Mascarenes, La Réunion and Mauritius, where the major vector, *A. variegatum*, is established (Fig. 13.7). Countries like Lesotho, Somalia, southern Angola, Botswana, Namibia, and western and south-central South Africa have not been threatened by heartwater since their climate is unsuitable for *Amblyomma* ticks (Yunker 1996). *A. variegatum* is also present in the Caribbean islands, and heartwater is endemic in Guadeloupe, Marie Galante and Antigua. According to OIE, global geographical areas with reported cases of heartwater see their size decreasing since 2010 (http://www.oie.int/wahis_2/public/wahid.php/Diseaseinformation/Diseasedistributionmap) (Fig. 13.7).

As *A. variegatum* proved to be able to establish itself after accidental introduction in areas where climate is suitable and where hosts of adults, i.e. ruminants, and mainly cattle, are present, like the islands of Ocean or Atlantic oceans, there is fear that infected ticks could be introduced, by various means, into the American mainland where it could settle. Other areas, in tropical Asia or in north-east Australia for example, would also be suitable for *A. variegatum* (Barre et al. 2010).

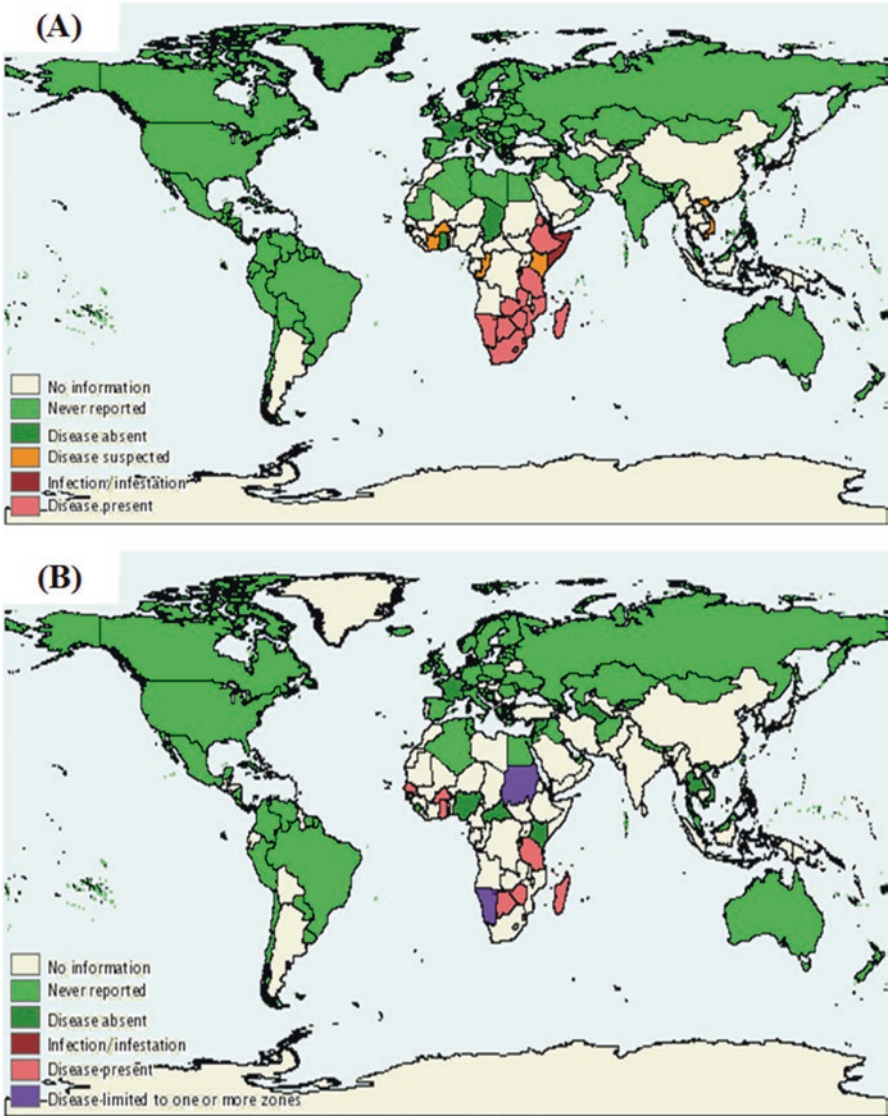

Fig. 13.7 Geographical distribution of Heartwater in the world (Source WAHID—OIE). (**a**) January to July 2010 and (**b**) January to July 2015

A. variegatum was introduced from West Africa into Guadeloupe and Martinique at the end of the eighteenth century and spread to Antigua in the nineteenth. Further spreading to other Lesser Antilles islands occurred at mid-twentieth century together with the spread of an erratic bird, the cattle egret, *Bubulcus ibis* (Barre and Uilenberg 2010). To control tick spreading in the Caribbean and reduce introduction danger to American mainland, several eradication programmes were created. From 1994 to 2008, the Caribbean *Amblyomma* programme (CAP) was implemented in the

English Lesser Antilles (Anguilla, Antigua and Barbuda, Barbados, Dominica, Montserrat, Nevis, St Kitts, St Lucia and Saint Maarten) to eradicate *Amblyomma* ticks (Ahoussou et al. 2010; Pegram et al. 2004). It allowed a decrease of tick infestation on six islands at the end of the project: Sainte Lucia, Saint Kitts, Montserrat, Anguilla, Dominica and Barbados. Currently, four islands have rare *Amblyomma* ticks (Saint Vincent and Saint Croix) or are *Amblyomma* free (Anguilla, Barbuda, Montserrat and Barbados; Fig. 13.8). In Dominica and Sainte Lucia, restricted area with high numbers of ticks (hot spots) are currently observed whereas Martinique, Saint Kitts and Nevis have low to moderate level of tick infestation. Another *A. variegatum* control programme targeting French islands was also implemented at the same time but with less success than CAP, resulting in a remaining high level of infestation in Guadeloupe and Marie Galante (Molia et al. 2008). Previous studies performed in these two islands indicated that the tick infested 35.6 % and 73.8 % of the herds in Guadeloupe and Marie Galante, respectively, with 36.7 % and 19.1 % of *A. variegatum* ticks infected with *E. ruminantium*, respectively (Molia et al. 2008; Vachiery et al. 2008a). Therefore, these islands constitute a reservoir for ticks and *E. ruminantium* in the Caribbean, threatening the American mainland through the spreading of infected *A. variegatum* nymphs by migratory birds or uncontrolled movement of animals (Kasari et al. 2010). If an accidental introduction of a tick-free *E. ruminantium* carrier animal would happen, autochthonous *A. maculatum* and *A. cajennense* (which have proven to be experimental vectors for heartwater) could promote *E. ruminantium* spreading from the North of Mexico down to the South of Brazil (except Andean region). Since 2012, heartwater has been identified within the 12 most important animal transboundary diseases for US (Vachiéry et al. 2013; Roth et al. 2013).

Although eradication programmes are now in standby in the Caribbean, heartwater control and early diagnostic are a major concern in Guadeloupe in parallel with the development of research programmes at international level to fill the gap of efficient vaccines and diagnostics. A surveillance network (RESPANG, surveillance network for nervous ruminant pathology in Guadeloupe) was also created from 2010 to 2015, in collaborations with veterinarians, French Ministry of Agriculture and the OIE reference laboratory for heartwater (CIRAD), in order to detect sick animals with heartwater suspicion, and to perform a sensitization campaign for farmers concerning acaricide treatment and recommendations.

4.2 Animal Species Affected

Small ruminants, goat and sheep, are more susceptible to heartwater than cattle. Moreover, there is also a variation between breeds: for instance, *Bos indicus* (zebu-type cows) breeds are generally more resistant than European breeds (Uilenberg 1983), not only because of enzootic stability. A wide variety of wild ruminant species may become infected with *E. ruminantium*, some showing symptoms (bleskbok, black wildebeest, giraffe, eland, etc.) and others not (buffalo, impala, greater Kudu). Other animals such as helmeted guinea fowl, leopard tortoise and scrub hare have

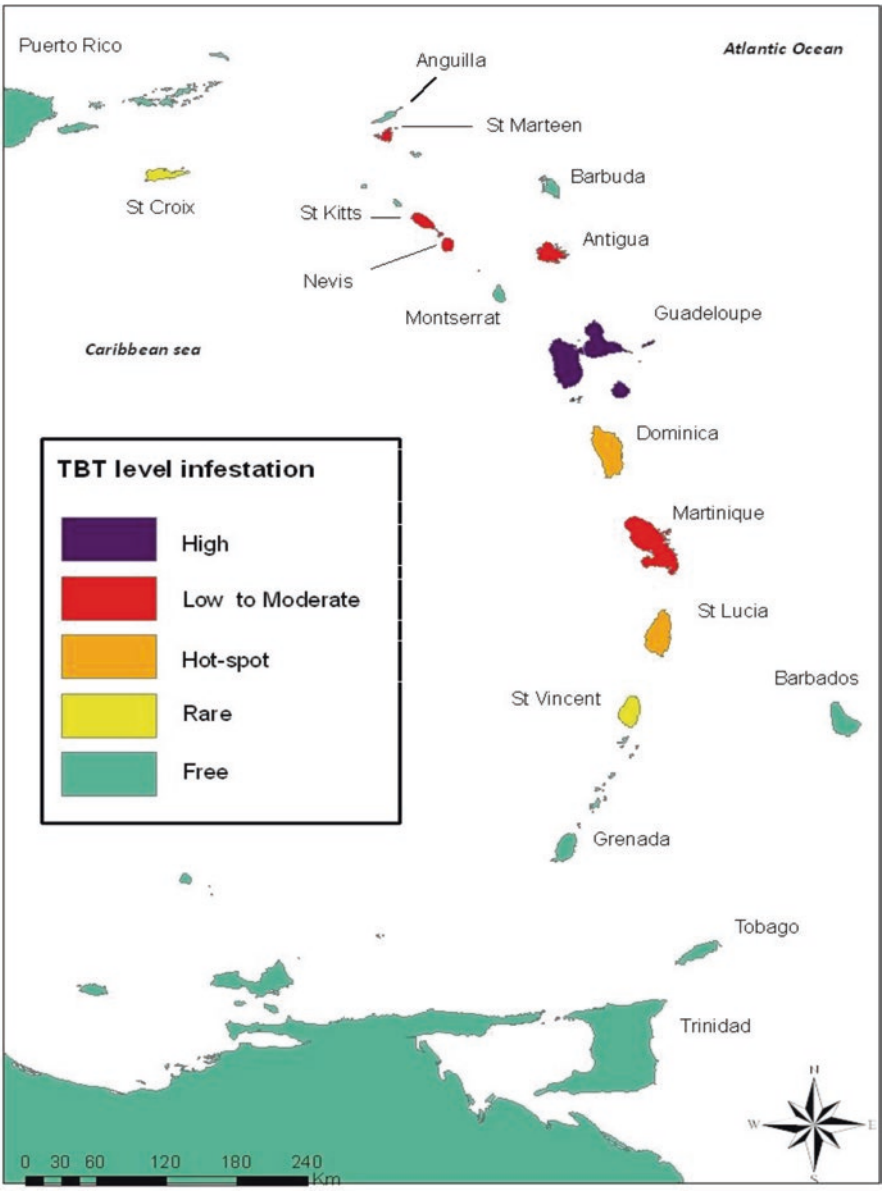

Fig. 13.8 *Amblyomma variegatum* infestation in Lesser Antilles, data from CaribVET network (2011)

been reported to develop sub-clinical heartwater (Oberem et al. 1987; Kock et al. 1995; Peter et al. 1999a). Knowledge of the susceptibility of wild ruminants to heartwater is particularly important where re-introduction of ruminant game species into heartwater endemic areas is considered. Wild ruminants also play a role as sources of infection for ticks, particularly in those areas where stringent tick control in domestic

animals is practiced (Peter et al. 1999b). Still the role of wildlife in the epidemiology of heartwater has not been elucidated (van Vuuren and Penzhorn 2015).

E. ruminantium can also infect ferrets and mice (Oberem and Bezuidenhout 1987). Although the mouse was used as an animal model for the characterization of immune responses during infection or for the development of vaccine candidates (Du Plessis et al. 1991; Nyika et al. 1998; Kock et al. 1998; Byrom et al. 2000a, b; Brayton et al. 2003; Simbi et al. 2006), very few *E. ruminantium* strains are known to infect mouse, limiting the use of this animal model for further studies.

Although there are no official records in the literature of heartwater disease in humans, three fatal cases of possible human infection by *E. ruminantium* have been reported in South Africa, revealed by genetic typing of *E. ruminantium* DNA sequences in brain tissue and serum samples associated with clinical features of heartwater such as pulmonary oedema and an oedematous, hyperaemic brain (Allsopp et al. 2005b, c). No confirmatory isolation of bacteria in culture and further genetic characterization were performed, and no new record of such infection was published since. Still heartwater is now considered as a tick-borne pathogen of potential zoonotic importance in the Southern African region (Esemu et al. 2011; Chitanga et al. 2014).

5 Diagnosis of Heartwater

Suspicion of heartwater occurs because of the recognition of clinical signs (essentially neurological damages changing the behaviour of the infected animals), presence of *Amblyomma* spp. ticks, and presence of transudates in the pericardium and thorax on post-mortem examination. The traditional method of diagnosis is the identification of the pathogen through post-mortem microscopic examination of brain smears, or bacterium isolation from infected blood or tick homogenates. Serological assays (indirect fluorescent antibody (IFA) test, enzyme-linked immunosorbent assays (ELISA) and immunoblotting (western blotting)) have been developed but suffer from poor sensitivity and specificity. Development of several *E. ruminantium* molecular diagnostics has been performed based on classical bacterial DNA amplification improved by nested and real-time PCR techniques.

5.1 Clinical Signs

The incubation period in natural infections is usually 2–3 weeks, but can vary from 10 days to 1 month, without any early clinical or physiological indicators, except that the rickettsemia coincides with the onset of fever. The course of the disease may range from the relatively rare peracute form (with sudden death without symptoms apart from high hyperthermia up to 42 °C) to mild, depending on age, immune status, breed and virulence of *E. ruminantium* strain (Van de Pypekamp and Prozesky

1987). The clinical signs may include a sudden fever, severe respiratory distress, hyperesthesia, lacrimation, terminal convulsions, and sudden death. Occasionally, animals also have diarrhoea. Animals with the acute form of heartwater are restless and show nervous symptoms such as rapid blinking of the eyes, hypersensitivity to touch, walking in circles, sucking movements, rigidly standing with tremors of the superficial muscles and finally, they fall to the ground, pedalling. They usually die within a week after the onset of the disease. Recovery is rare when nervous symptoms have started (Van de Pypekamp and Prozesky 1987; OIE 2005). Immune animals may have small transient hyperthermia and natural rapid recovery, or even no sign of infection.

5.2 Pathogenesis

The pathogenesis of the disease, despite still poorly understood, remains of help in confirming the suspicion of heartwater. Increased vascular permeability results in transudation of fluid into various body tissues (e.g. brain and lungs) and body cavities (e.g. pericardial and thoracic cavities) (Fig. 13.9), but the precise mechanisms responsible for the transudation are poorly understood. At necropsy, it is possible to observe hydro-pericardium (origin of the name "heartwater"), with straw-coloured to reddish pericardial fluid; this phenomenon appears to be more pronounced in sheep and goats than in cattle (van Amstel et al. 1988; Van Amstel et al. 1987; Brown et al. 1990). Brain oedema leads to nervous signs, hydropericardium contributes to cardiac dysfunction during the terminal stages of the disease, and progressive pulmonary oedema and hydrothorax result in asphyxiation (Uilenberg 1971; Owen et al. 1973). The pathogenesis of vascular permeability remains speculative as the intracytoplasmic development of the organisms (reticulate bodies) seems to have little detectable cytopathic effect upon the endothelial cells (Pienaar 1970), and there is also no apparent correlation between the number of parasitized cells in the pulmonary blood vessels and the severity of the pulmonary oedema (Prozesky and Du Plessis 1985). It has been proposed that an endotoxin (Amstel et al. 1988) and increased cerebrospinal fluid pressure (Brown and Skowronek 1990) play a role in the development of lung oedema.

5.3 "Brain Squash Smears"

In clinical cases, heartwater must be differentiated from a wide range of infectious and non-infectious diseases, especially plant poisonings, which also cause central nervous system signs. For acute clinical cases in endemic areas, clinical signs alone may suggest the aetiology, but demonstration of the organism in the cytoplasm of capillary endothelial cells is necessary for a definitive diagnosis. Typical colonies of *E. ruminantium* can be observed in "brain squash smears" made after death of the

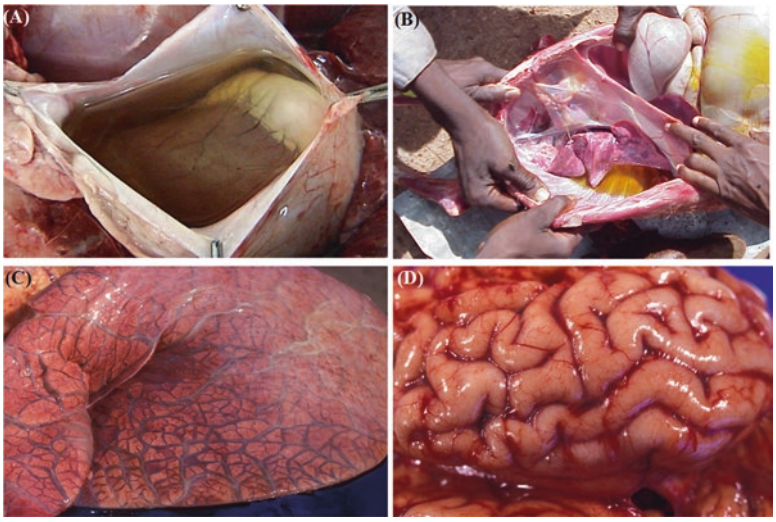

Fig. 13.9 Lesions due to *Ehrlichia ruminantium* infection in the ruminants. (**A**) Oedematous pericardium of a goat died of heartwater disease; (**B**) Severe hydrothorax in cattle dead of heartwater; (**C**) Severe lung oedema associated with heartwater (bovine); (**D**) Brain oedema associated with heartwater (bovine) (adapted from Allsopp et al. (2005a)), photos (**A**) and (**B**) are from Ken Giraud-Girard and Frédéric Stachurski (both from CIRAD)

infected animal. For this, a piece of gray matter from the cortex (~3 × 3 mm) is crushed between two microscope slides; the softened material is then spread like a blood smear with the material pushed rather than pulled along. A slight lifting of the spreader slide about every 5–10 mm creates several thick ridges across the slide, from which capillaries are arranged straight and parallel in the thin sections of the smear for easier examination. Brain smears are then air dried, fixed with methanol and stained with Eosine and methylene blue (RAL555) or Giemsa and observation of *E. ruminantium* is then possible (Fig. 13.2).

E. ruminantium occurs as clumps of reddish-purple to blue, coccoid to pleomorphic organisms in the cytoplasm of capillary endothelial cells close to the nucleus (Fig. 13.2). Colonies can be difficult to find in some samples depending on the *E. ruminantium* strain, in samples from animals treated with antibiotics or from animals with peracute disease. Colonies are still visible 2 days after death in a brain stored at room temperature (20–25 °C) and up to 34 days in a brain stored at 4 °C. Technical expertise is required to differentiate *E. ruminantium* colonies from other haemoparasites (such as *Babesia bovis*), certain blood cells (thrombocytes, granulocytes), normal subcellular structures (mitochondria, mast cell granules), or stain artefacts (stain precipitates).

To improve the histological diagnosis, an immunohistochemical staining technique was developed using a specific serum targeting the major antigenic protein-1 (MAP-1), but is now rarely used. In naturally infected cattle, sheep and goats, *E. ruminantium* morula are identified in formalin-fixed tissues as clearly defined,

brown-staining rickettsial colonies within the cytoplasm of endothelial cells, whereas no positive staining is observed in the control group (Jardine et al. 1995).

5.4 Serological Tests

Two serological diagnostics based on the detection of antibodies against *E. ruminantium* MAP-1protein are currently used: a competitive ELISA MAP1 and an indirect ELISA using a fraction of MAP1 protein, MAP1-B (van Vliet et al. 1995; Katz et al. 1997). Unfortunately, these assays display cross-reaction with other *Ehrlichia* species, specifically with *E. chaffeensis* and *E. muris*. The indirect MAP1-B ELISA is used routinely at the regional OIE reference laboratory for heartwater (CIRAD). ELISAs for heartwater diagnosis are suitable for prevalence studies at herd level but cannot be used either for specific diagnostic purposes on clinical cases or to evaluate the infectious status of imported animals. Indeed, there is a 15-day delay in seroconversion after animal infection, and the seropositivity period lasts only several weeks for bovines and less than 6 months for small ruminants, whereas animals remain immune and possibly reservoir of the pathogen for months and sometimes up to 2 years after infection. Moreover, according to the current knowledge, seropositivity to *E. ruminantium* appears to be asynchronous with the infectious kinetics as a whole, as well as with the status of immunocompetent animals. Serodiagnosis of animals previously exposed to the disease, i.e. recovered from subclinical or clinical infection, still poses problems.

5.5 Molecular Diagnosis

Molecular diagnostics are the gold standards for the diagnosis of heartwater. In the last 15 years, important improvements have resulted in the development of better molecular tools for the diagnosis as well as genetic typing of different strains of *E. ruminantium*.

5.5.1 PCR and Nested PCR

The molecular method consisting in PCR amplification of a Crystal Springs strain *pCS20* DNA fragment, specific of *E. ruminantium* (GenBank accession number X58242), followed by membrane hybridization has been first developed (Mahan et al. 1992). Low levels of infection in animals and in ticks fed on carrier animals are detected by PCR, while a hybridization reaction with the *pCS20* probe alone (without PCR first) usually remains negative (Peter et al. 1995). Experimentally, the detection limit of the conventional PCR assay was found to be between 10 and 10^2

organisms per sample, whereas it was between 1 and 10 organisms after PCR/hybridization. Thirty-seven strains from all endemic areas have been detected by PCR/hybridization with a high specificity (98 %). However, the sensitivity of the PCR assay is variable depending on the sample nature and *E. ruminantium* load (Peter et al. 2000).

Two nested PCR assays were developed to enhance the detection of low levels of rickettsemia (Semu et al. 2001; Martinez et al. 2004); both use the *pCS20* region as the target sequence. The method defined by Semu and co-workers (Semu et al. 2001) is based on two external primers U24 and L24 primers for the first amplification step followed by primers AB 128 and AB 129 as the nested primers, which amplify a 279-bp fragment from within open reading frame 2 of the 1306-bp pCS20 DNA sequence (Peter et al. 1995). The sensitivity of detection of this assay is one organism per sample. The other nested PCR assay uses a pair of external primers and comprises the AB128 sense primer together with an anti-sense primer called AB130 (Martinez et al. 2004). These amplify a 413 bp fragment used as a template in a second round PCR using also AB128 and AB129 as internal primers. The nested PCR shows an average detection limit of six organisms per sample (Martinez et al. 2004). The *pCS20* nested PCR allowed regular detection of *E. ruminantium* organisms from ticks, blood, brain and lungs from infected animals, whether the samples were processed fresh, after freezing or preservation in 70 % ethanol. Presently, the molecular diagnosis is much more sensitive and faster than histological or microbiological diagnosis. For example, brain smear observations and pCS20 nested PCR on the same brain samples demonstrated a higher percentage of heartwater positive cases that increased from 75 % based on brain smears observations to 97 % using *pCS20* nested PCR (Adakal et al. 2010).

The range of strain detection was later on increased by the use of new primers AB128' AB130' and AB129' modified from the original AB128, AB129 and AB130 by including universal nucleotides and this method is used routinely for *E. ruminantium* detection in field samples, especially in ticks (Molia et al. 2008; Adakal et al. 2009, 2010b). The detection of *E. ruminantium* by nested PCR is possible in the blood of animals 1 or 2 days before hyperthermia and during the hyperthermia period but not on asymptomatic animals. PCR-based methods appear to be more reliable in detecting infection in ticks and this could have epidemiological value in determining the *E. ruminantium* prevalence in ticks and the geographical distribution of *E. ruminantium*.

A nested PCR targeting the entire *map1* polymorphic gene has been developed in parallel in order to type the strains by restriction fragment length polymorphism (RFLP) or sequencing of the amplification fragment directly from the *pCS20* positive samples (Martinez et al. 2004). Its detection limit was evaluated at around 60 organisms per sample.

The main drawback of nested PCRs is the possibility to get cross-contamination due to second round of PCR and, technical cautions should be implemented to limit this risk particularly when manipulating the first PCR product with a high quantity of *pCS20* targeted gene. Moreover, this method is also time-consuming particularly for screening a large amount of samples.

5.5.2 Quantitative Real-Time PCR

Several quantitative real-time PCR have been developed for the detection of *E. ruminantium* targeting *map-1*, *map1-1* and *pCS20* genes (Peixoto et al. 2005; Postigo et al. 2002; Steyn et al. 2008). These methods have been described for the detection and quantitative determination of *E. ruminantium* organisms either for *E. ruminantium* kinetics in the blood of experimentally infected sheep (during the hyperthermia reaction period) (Steyn et al. 2008) or in vitro (Marcelino et al. 2005, 2006, 2007; Peixoto et al. 2005, 2007). Real-time PCR targeting *map1* and *map1-1* polymorphic genes were tested on a limited number of strains (up to six), and therefore, should not be used for diagnostics. Another real-time PCR assay targeting *pCS20* gene has a sensitivity level similar to the nested PCR, but asit was tested so far only on 15 different strains, screening on additional strains should be performed before using as diagnostic tools. Recently, Sayler and coworkers (Sayler et al. 2016) developed and validated a dual-plex Taqman QPCR assay targeting the *groEL* gene of Panola Mountain *Ehrlichia* and *E. ruminantium*. Twenty-three *E. ruminantium* strains originated from 12 countries (from Africa and Caribbean) were detected but *E. chaffeensis*, *E. ewingii* or *E. canis*, or *Anaplasma* spp. were not detected. It represents a promising method compared to nested PCR due to limitation of cross-contamination, but the strain recognition spectra need to be enlarged to validate the proof of concept before using it as a gold standard molecular diagnostic tool.

5.5.3 Typing Methods

The genetic characterization and structure of *E. ruminantium* population at regional scale is essential in order to select potential vaccine strains. The genetic typing of strains was previously done using RFLP on the polymorphic gene *map-1* after PCR amplification (Faburay et al. 2007; Adakal et al. 2010). Based on the genome analysis of two different strains, Gardel and Welgevonden, truncated and unique coding sequences specific of strains have been identified. This analysis allowed the development of a differential strain-specific diagnosis using nested PCRs targeting six unique and four truncated CDS (Vachiery et al. 2008b). New multi-locus methods adapted to *E. ruminantium* have been validated such as multi-locus sequence typing (MLST) (Adakal et al. 2009, Nakao et al. 2011, Cangi et al. 2016) and multi-locus variable number of tandem repeated sequence analysis (MLVA) (Pilet et al. 2012). Two studies on restricted areas in Burkina Faso demonstrated the presence of several different clusters and identified one strain population in stasis and another strain population in clonal expansion. Cangi and co-workers recently used MLST to analyse the genetic diversity and population structure of 194 *E. ruminantium* strains circulating worldwide. This study highlighted the importance of recombination events in the generation of *E. ruminantium* diversity and evolution and revealed that the strains were clustered into two major genetic groups: a West African cluster, and a worldwide cluster which includes West Africa, East Africa, Southern Africa, Indian Ocean, and Caribbean (ref: Cangi, N., Gordon, J. L., Bournez, L., Pinarello,

V., Aprelon, R., Huber, K., … Vachiéry, N. (2016). Recombination Is a Major Driving Force of Genetic Diversity in the Anaplasmataceae Ehrlichia ruminantium. Frontiers in Cellular and Infection Microbiology, 6, 111. http://doi.org/10.3389/fcimb.2016.00111)

With the important progress in the performances of sequencers (Illumina or PGM Ion torrent) since 2010, sequencing of whole genome of *E. ruminantium* strains is now possible in a very short time allowing getting information on all genes. However, this method is restricted to samples produced in cell culture as field samples (ticks of blood of infected animals) do not contain enough *E. ruminantium* organisms for the analyses.

5.6 *Identification of Suitable Diagnostic for Heartwater Diagnosis*

A comparison of the different molecular diagnostic methods for heartwater is presented in Table 13.1. The nested PCR *pCS20* presents several advantages and it is the gold standard assay for diagnostic of clinical heartwater specimens. Nevertheless, the real-time PCR *pCS20* is faster and less prone to cross-contamination. It is thus essential to do further validation and compare the detection efficiency between real-time and nested PCR *pCS20* in order to validate the real-time PCR assay for heartwater diagnosis.

A molecular multi-pathogen method, the reverse line blot (RLB), is also available, targeting *Ehrlichia* and *Anaplasma* sp. including *E. ruminantium*, *A. margi-*

Table 13.1 Comparative analysis of the available molecular tools for heartwater diagnosis (Vachiery et al. 2013)

Critical criteria	Nested PCR *pCS20*	qPCR *map1*	qPCR *map1-1*	qPCR *pCS20*	Research needs
Rapidity	5	8	8	8	Development of quick tests: Multi-pathogen detection by microarrays or qPCR
Sensitivity	8	6	5	8	
Handling and caution to avoid cross-contamination	5	8	8	8	
Detection of different ER strains	10	2	2	3	Comparison of detection capacity between *pCS20* nested PCR and qPCR
Adapted to field samples	10	NT	5	5	
Total score	38	24	29	32	

NT not tested
Scoring from 0 to 10, where 0 = the worst and 10 = the best

nale, A. centrale, A. ovis and *A. phagocytophilum* (Bekker et al. 2002). Nevertheless, it is less sensitive than the nested PCR and it has not been validated at large scale and during epidemiological studies. There is therefore a strong interest and usefulness in developing improved rapid multi-pathogen detection assay, i.e. using microarray technology or multiplex qPCR as done by Sayler and coworkers (Sayler et al. 2016) and that could also include other tick-borne pathogens such as *Babesia* and *Theileria* spp.

6 Treatment, Control and Prevention

As mentioned above, in enzootic areas, indigenous cattle have developed resistance to heartwater due to long-term interaction with the bacteria. Although natural enzootic stability should be considered as the desirable situation where no control measures would need to be implemented, this stability can be easily disrupted by variations in climate, host and pathogen phenotypes, and management strategies (Florin-Christensen et al. 2014). As enzootic stability can be sometimes difficult to achieve, several strategies are developed to control heartwater. These include chemotherapy and chemoprophylaxis, vector chemical control and vaccination.

6.1 Chemotherapy and Chemoprophylaxis

Treatment with antibiotics (tetracyclines) of infected animals during the early febrile stages is very efficient and confers long-lasting immunity. The main problem is the timing of treatment of field cases: in general, animals display visible (nervous) symptoms and are presented for treatment when it is too late to treat.

In goats, it was proposed to use short-acting tetracyclines administered at a dosage rate of 3 mg/kg body weight on 10, 20, 30, 45 and 60 days after introduction in an endemic area, during the nymph infestation periods, to allow acquisition of protective immunity. In that case, animals should not be dipped during all the immunization procedure (Infection and treatment method, see below) (Gruss 1981). Similarly, injections of long-acting tetracycline formulations (10–20 mg/kg body weight) given on days 7, 14 and 21, or even on only two occasions (days 7 and 14) in cattle are sufficient to protect them from contracting heartwater, while at the same time allowing them to develop a natural immunity, when introduced in heartwater endemic regions during peak infestation level by nymph or adult ticks (Purnell 1987). The success of this regime is of course dependent on all the animals becoming naturally infected with heartwater during the time that they are protected by the drug: time of introduction and treatment has to be determined according to seasonal variations of ticks.

6.2 Vector Chemical Control

Heartwater is usually introduced into free areas by infected animals, including subclinical carriers, or by infected ticks. Sustained and intensive tick control measures may thus succeed in preventing outbreaks of heartwater in tick-free areas, and even, under certain conditions, in enzootic areas. The disease can however only be controlled successfully if all the animals on the farm are treated regularly with acaricides throughout the year and if there are no, or an absolute minimum, of game animals and birds on which ticks can survive. Because of the high infection rate of the vector ticks, it is nevertheless very difficult and expensive to prevent heartwater by vector control in enzootic areas. Moreover, the use of acaricides also has a negative impact on environment and human health. Although tick populations resistant to acaricide have only been very rarely found in *Amblyomma* spp. ticks, it is moreover considered that high frequency treatment programmes may lead, sooner or later, to the development of tick resistance most probably on other tick species such as *Rhipicephalus microplus* to the used compound. Such intensive programmes can also lead to disappearance of enzootic stability, even in local cattle breeds.

6.3 Vaccination

Four different vaccine strategies against heartwater have been developed: the "infection and treatment" method using live bacteria, infection with in vitro attenuated bacteria, immunization with inactivated in vitro grown bacteria and recombinant or subunit (using DNA or recombinant proteins, respectively) (Table 13.2). For all these vaccines, the main problem is the presence of numerous strains in the field with high genetic and/or phenotypic diversity (as above mentioned) and, sometimes, the reduced level of cross-protection between them. The choice of the vaccine strain(s) is therefore crucial and depends on the region, as it was previously demonstrated by Adakal and co-workers (Adakal et al. 2010).

6.3.1 The "Infection and Treatment" Method

Field observations and experiments under laboratory conditions have shown that cattle, sheep and goats are capable of developing a protective immunity against heartwater after surviving a virulent infection. In South Africa, this led to the development of an "infection and treatment" type of immunization where animals are injected with fully virulent *E. ruminantium* organisms of the Ball 3 strain and are subsequently treated at onset of hyperthermia with tetracyclines to prevent disease, which requires daily monitoring of body temperature (du Plessis and Bezuidenhout 1979). Despite the low cross-protection of the Ball 3 strain against some other *E. ruminantium* strains and the fact that this is an expensive and dangerous

Table 13.2 Examples of vaccination strategies tested for *Ehrlichia ruminantium* infection

Type of vaccine	Host	Vaccine isolate	Challenge isolate[b]	Survival ratio (%)	References
Live	Cattle	Ball 3	Ball 3	100	du Plessis and Bezuidenhout (1979)
Attenuated	Sheep	Senegal	Senegal, Welgevonden, Umpala, Lutale, Ball 3, Gardel, Um Banein	0–100	Jongejan (1991), Jongejan et al. (1993)
	Sheep	Welgevonden	Wegevonden, Ball 3, Gardel, Mara 87/7, Blaauwkrans	100	Zweygarth et al. (2005), Collins et al. (2003a)
	Goat	Welgevonden	Welgevonden	80	
Inactivated cell cultured organism (+ adjuvant)	Goats	Gardel	Gardel	65 70–100	Martinez et al. (1994, 1996), Vachiery et al. (2006), Marcelino et al. (2015a, b), Marcelino et al. (2007)
	Sheep	Crystal Spring	Crystal Spring	100	Mahan et al. (1995, 1998a, b)
			Crystal Spring (ticks) Beatrice (ticks)		Mahan et al. (1998a)
		Mbizi	Mbizi, Beatrice, Isiolo, Welgevonden	60–100	
		Mbizi	Beatrice (ticks)	70	
	Cattle	Gardel	Gardel	100	Totte et al. (1997)
	Goats/Sheep/Cattle	Mbizi, Sunnyside, Lutale, Bathurst	Field tick challenge	26–80	Mahan et al. (2001)

(continued)

Table 13.2 (continued)

Type of vaccine	Host	Vaccine isolate	Challenge isolate[b]	Survival ratio (%)	References
Recombinant (DNA and/or recombinant protein)	Mice	*map-1* DNA vaccine (Crystal Spring)	Crystal Spring	25–90	Nyika et al. (1998); Nyika et al. (2002).
	Sheep	Cocktail of genes (Welgevonden)	Welgevonden	20–100	Collins et al. (2003a, b), Pretorius et al. (2002, 2007, 2008, 2010)
	Mice	*cpg1* gene (Welg)	Welgevonden	0	Louw et al. (2002)
	Sheep			80	
	Mice	*E. coli* lysates expressing 5 different genes	Highway	Highly variable	Barbet et al. (2001)

methodology, it has been the only commercially available vaccine strategy for more than 50 years (Du Plessis et al. 1989, Onderstepoort Biological Products SOC Ltd)). Whenever large numbers of commercial ruminants of high value are introduced to heartwater endemic regions, the block method of vaccination is also used: after vaccine administration, the animals are treated simultaneously and indiscriminately with antibiotics whether a febrile reaction occurs or not (Du Plessis and Malan 1987). This method has many drawbacks such as the requirements of a cold chain, a short shelf-life and the potential for the transmission of other pathogens and could not be used widely (Shkap et al. 2007).

6.3.2 Live Attenuated Vaccine

In the early 1990s, an attenuated strain of *E. ruminantium* (Senegal) was prepared as a live vaccine by serial passage in vitro in endothelial cells (Jongejan 1991). This attenuated strain, while providing immunity to homologous challenge, was nevertheless not fully effective to provide cross-protection against other virulent strains (Jongejan et al. 1993) (Table 13.2). Another strain from Guadeloupe (Gardel) can also be attenuated after 200 passages in endothelial cells (Marcelino et al. 2015b, Martinez 1997). This strain provides a good protection against heterologous challenge with other strains (Martinez 1997). Zweygarth and co-workers have successfully attenuated the virulent Welgevonden strain of *E. ruminantium* by 50 continuous passages in a canine macrophage-monocyte cell line (Zweygarth and Josemans 2001). The use of such live attenuated vaccines is nevertheless limited since cross-protection against different isolates is not complete. In comparison with other immunization methods, the main disadvantage of attenuated vaccines is the possible reversion to virulence and the need to storage in liquid nitrogen until used.

6.3.3 Inactivated Vaccine

The inactivated vaccine is based on purified *E. ruminantium* organism (produced in bovine endothelial cells) that are chemically inactivated or lysed (Martinez et al. 1994, 1996; Mahan et al. 1998a). In 2006, Marcelino and co-workers developed a fully scalable process for the large-scale production of the antigen using bioreactors and microcarriers; this bioprocess also allows to decrease the price of a vaccine dose (Marcelino et al. 2006). The development of a large-scale purification process using membrane (Peixoto et al. 2007) and theoptimization of theantigen buffer also improved the level of purity of the vaccine and its storage conditions (Marcelino et al. 2007). Field tests proved however that a strain isolated in a region is less effective when used elsewhere, and that local strains should be added to improve vaccine effectiveness (Adakal et al. 2010).

In 2015, Marcelino and co-workers developed a ready-to-use inactivated vaccine that could be easily used in the field and even withstand up to 3–4 days at 37 °C before injection (Marcelino et al. 2015a). As soon as regional isolates would be

available in culture after isolation, it could be possible to produce an inactivated vaccine including a cocktail of regional strains. The main difficulty is to choose the strains which could protect against other circulating strains. The choice will depend on genetic characteristics and markers, which are not yet precisely defined. The main inconvenient of the inactivated vaccines is the observation of animal morbidity during the infectious challenge.

6.3.4 Recombinant Vaccine

Besides the increased safety and reduced price, the use of recombinant vaccines could permit the correct presentation of the antigen after endogenous processing leading to a long-lasting immunity. To develop such a vaccine, it is nonetheless necessary to identify *E. ruminantium* antigens that would induce a protective immune response. The *map1* gene was cloned and tested as a naked-DNA vaccine in a mouse model system (Nyika et al. 1998, 2002). Others genes such as groE operon (*groES* and *groEL)* (Lally et al. 1995) and *cpg 1* (Louw et al. 2002) have also been cloned and tested as a recombinant DNA vaccine to protect animals against lethal *E. ruminantium* infection (van Vliet et al. 1993, 1994, 1995, 1996; Reddy et al. 1996; Mahan et al. 1994; Nyika et al. 1998, 2002) (Table 13.2). Subunit vaccines using denatured *E. ruminantium* have also been tested, although no protection was achieved (van Vliet et al. 1993). Since 2007, Pretorius and co-workers evaluated the ability of several *E. ruminantium* ORFs as an efficient component of a recombinant vaccine against heartwater (Pretorius et al. 2007, 2008, 2010). When the authors immunized the animals either with a recombinant DNA cocktail of four 1H12 pCMViUBs_ORFs followed by a r1H12 protein or with 1H12 plasmid rDNA, a survival ratio of 100 % against a virulent *E. ruminantium* Welgevonden needle challenge was obtained (Pretorius et al. 2007). When a similar strategy was used but using a tick challenge, only 20 % of protection was obtained (Pretorius et al. 2008). A prime/boost vaccination trial using the polymorphic *cpg1* gene and the recombinant protein also resulted in complete protection of vaccinated animals after homologous challenge; no trials with heterologous strains have yet been performed (Pretorius et al. 2010). Due to the polymorphic property of *cpg1*, a cocktail of representative *CpG1* from different strains should be included in the vaccine before any field trial. In the same year, Sebatjane and co-workers also tested five *E. ruminantium* ORFs (Erum7340, Erum7350, Erum7360, Erum7380, and Erum4360) coding for membrane proteins of low molecular weights as potential antigen against heartwater (Sebatjane et al. 2010). Unfortunately, the vaccination experiment in sheep using a DNA/protein prime/boost resulted in a low survival ratio (20 %).

From the above mentioned, recombinant vaccines look promising under experimental conditions, but results during field trials have been less successful. Moreover, simple intramuscular immunization is not sufficient to induce protection, and the use of a gene gun necessary for prime DNA injection is not suitable for large field vaccination campaign.

6.3.5 Identification of theBest Vaccine Currently Available

A comparison between the different vaccines is presented in Table 13.3. This analysis is based on critical criteria: efficacy against homologous/heterologous strains, safety, induction of low morbidity, availability of industrial process, stability, supply, easiness of administration and ability to elaborate a regional vaccine. At the moment, the inactivated vaccine seems to be the most appropriate for worldwide vaccination strategy against heartwater since: (1) a bioprocess is already optimized, (2) it includes killed bacteria, (3) storage condition is compatible with field use and (4) it is suitable for regional cocktail of strains to improve vaccine efficacy. .

The diversity of *E. ruminantium* strains remains the main problem for all candidate vaccine mentioned above. For instance, only one Caribbean strain is currently available in culture; it is thus necessary to isolate and cultivate new Caribbean strains to prepare a vaccine cocktail that will be suitable for all the regional area. This will

Table 13.3 Comparative analysis of different vaccines currently available for heartwater (adapted from Vachiery et al. 2013)

Critical criteria	Infection and treatment	Recombinant vaccine	Attenuated vaccine	Inactivated vaccine	Research needs
Efficacy/ homologous challenge	10	10	10	10	
Efficacy/ heterologous challenge	5	**2**	5	5	Genetic and phenotypic characterization
Safety	**0**	8	**3**	8	Knowledge of virulence mechanisms[a]
Low morbidity	**0**	8	8	3	
Time to availability	7	7	4	6	
One dose	10	**2**	8	4	
Industrial process	**0**	**0**	**0**	10	Development of bioprocess[b]
Stability/shelf life	8	NT	8	7	
Storage Distribution Supply	**0**	**3**	**0**	8	
Administration	**3**	**2**	**3**	8	
Regional vaccine	**0**	6	**0**	6	Development of regional vaccine: isolation of live Caribbean strains
Total score	43	48	49	**75**	

NT not tested
[a]Only for attenuated vaccine
[b]Graded on 1–10 scale, where 0 = the worst and 10 = the best

also be essential at the African continent level, to characterize and study genetic and antigenic features and compare them, in order to define efficient cocktails for vaccination against heartwater and their corresponding geographic areas of use.To understand the structure of the rickettsia population,the genetic characterization of some of the strains circulating in the field in Africa and Caribbean areas has been performed as mentioned above(Adakal et al. 2010; Pilet et al. 2012) Still, further improvements and experiments are required. Indeed,Cross-protection studies were performed at the OIE reference laboratory for heartwater (CIRAD) in attempt to link genotyping to cross-protection, but the correlation has proven to be somewhat difficult to establish (unpublished results). Still, these epidemiological molecular studies could help to choose for optimal vaccine strains as previously shown (Adakal et al. 2010).

Despite the efforts to develop an effective recombinant vaccine, few genes and/or recombinant proteins have been tested so far (Table 13.2). This might be due to the difficulty of selecting the best genes as until now little knowledge on virulence mechanisms is available. To overcome this issue, virulent and attenuated *E. ruminantium* strains from geographical distinct regions are being compared using high-throughput "Omics approaches" such as genomics, transcriptomics and proteomics(discussed in more detail in the following sections).

7 Immune Response to *Ehrlichia ruminantium* and Against Heartwater

The knowledge of protective immunity to *E. ruminantium* is still fragmentary. However, significant advances have been made towards the understanding of the mechanisms underlying antibody and cell-mediated immune response to this pathogen.

High antibody titres are normally detected in infected animals at the height of the febrile reaction, and this has led to the initial hypothesis that a humoral response might be involved in protection against heartwater (Semu et al. 1992). However, transfer of immune serum or gamma globulins from immunized to naïve animals have failed to protect animals or even modify the course of the disease (Du Plessis et al. 1984; Prozesky 1987b; Alexander 1931; Du Plessis 1993). Although these results show the limitations of using immune sera to induce protection, the possible existence of protective antibodies should not be fully excluded. Indeed, antibodies may be crucial in opsonization, complement-mediated killing and antibody-dependent cell-mediated toxicity and therefore deserve further investigations.

The apparent lack of an effective humoral response together with the report of a T-cell-mediated response in experimentally infected mice (Du Plessis 1982) have then led to the belief that immunity against heartwater is likely to be mediated by cellular responses directed against infected cells (Stewart 1987). *E. ruminantium* has a profound effect on endothelial cells. In vitro, it elicits the synthesis of IL-1β, IL-6 and IL-8 mRNA in infected host cells, and this effect appears to be potentiated by IFN-γ (Bourdoulous et al. 1995). IL-1 and IL-6 can act as co-stimulatory signals for T- and B-cells activation, while IL-8 might participate in the recruitment of neu-

trophils towards brain endothelial cells with potentially deleterious effect. Additionally, infection of endothelial cells with *E. ruminantium* strongly affect the expression of IFN-γ-induced MHC I and MHC II molecules at the surface of the host cells (Vachiery et al. 1998). Therefore, endothelial cells may have a pivotal role in the development of a protective immune response against heartwater.

Further experiments have shown that an array of molecular and cellular effectors is involved and that different responses can be obtained according to the immunization strategy (infection and treatment method, inactivated vaccine and recombinant vaccines). To understand which cell subsets and antigens are involved in the immune response against heartwater, in vitro lymphocyte proliferation tests were performed. When PBMCs obtained from live vaccines immunized cattle were stimulated with the two immunodominant recombinant antigens of *E. ruminantium* (MAP1 or MAP2), T-cell responses specific to MAP1 and MAP2 were generated. Proliferation of PBMCs was also elicited in vitro by infected, autologous endothelial cells, and *E. ruminantium*-primed monocytes, but not by killed organisms (Mwangi et al. 1998a). These proliferative responses were characterized by a mixture of $CD4^+$, $CD8^+$ and γδ T cells and strong expression of IFN-γ, tumour necrosis factors alpha and beta (TNF α/β), and interleukin-2 (IL-2), all which are strong indicators of a Th1-driven immune response. When PBMCs from animals immunized with inactivated antigens, $CD4^+$ T cells and IFN-γ were generated in response to *E. ruminantium* lysates and to *E. ruminantium*-primed autologous monocytes (Totte et al. 1997). These cell lines were also able to proliferate in vitro when stimulated with soluble *E. ruminantium* proteins between 20 and 32 kDa, previously fractionated by fast-performance liquid chromatography (FPLC) (Totte et al. 1998b) but they did not respond to the two immunodominant recombinant antigens of *E. ruminantium* (MAP1 or MAP2) (Totte et al. 1998a). Thus, during infection with live virulent *E. ruminantium*, T cell responses may be preferentially directed against certain epitopes expressed by infected cells but absent from the elementary body, the free extracellular stage of the organism (Fig. 13.5) (Totte et al. 1999). Flow cytometric analysis of PBMCs also showed that during vaccination experiments with inactivated vaccines, no significant change in the immune cell population occurred. However, after the challenge with virulent *E. ruminantium*, significant alterations were observed. After an initial progressive depletion of $CD4^+$, $CD8^+$ and T-cell subsets, an impressive rise in $CD8^+$ cells was observed (Martinez 1997; Mwangi et al. 1998b). This last finding is in accordance with the previous studies which led the authors to suggest that $CD8^+$ T cells might play a major role in immunity to heartwater described by Du Plessis with a murine model (Du Plessis 1982; Du Plessis et al. 1991, 1992).

In another approach, a naked-DNA vaccine containing the *map1* gene of *E. ruminantium* was used (Nyika et al. 1998). Immunized DBA/2 mice produced antibodies against MAP1 antigen and elicited a Th1 response, characterized by the production of IFN-γ and IL-2 in supernatant of splenocyte cultures stimulated with *E. ruminantium* lysates or recombinant MAP1 antigen. In 2010, Sebatjane and co-workers performed a DNA prime-protein boost immunization in sheep based on low molecular weight (LMW, <20 kDa) proteins of *E. ruminantium*, and confirmed the importance of sustained IFN-gamma production in conferring a protective immunity against heartwater (Sebatjane et al. 2010)

8 "Omics" Approaches for Improved Understanding of *E. ruminantium* Infection and Pathophysiology

Global "Omics" approaches (genomics, proteomics, transcriptomics, and metabolomics) in a systems biology context are becoming key tools to increase knowledge on the biology of infectious diseases, specially to improve knowledge of the complex host–vector–pathogen interactions (Marcelino et al. 2012b) (Fig. 13.10). These interactions consist of dynamic processes involving genetic traits of hosts, pathogens, and ticks that mediate their development and survival (Popara et al. 2015). .Nowadays, three complete *E. ruminantium* genomes (Frutos et al. 2006a, b; Collins et al. 2005) are available and the sequencing of three *new E.ruminantium* strains is being performed Nakao et al. (2016). This overall genomic information paves the way of using "Omics" approaches for this pathogen.

In 2009, Emboulé and co-workers optimized the Selective Capture of Transcribed Sequences (SCOTS) methodology to successfully capture *E. ruminantium* mRNAs, avoiding the contaminants of host cell origin and eliminating rRNA which accounts for 80 % of total RNA encountered (Emboule et al. 2009). This method is essential to perform transcriptomic studies on the intracellular form of the bacterium (reticulate body, RB) avoiding host cell contaminants. In 2011, Pruneau and co-workers (Pruneau et al., 2011) determined the genome-wide transcriptional profile of *E. ruminantium* replicating inside bovine aortic endothelial cells (BAECs) using cDNA microarrays. Interestingly, over 50 genes were found to have differential expression levels between RBs and EBs. A high number of genes involved in metabolism, nutrient exchange and defence mechanisms, including those involved in resistance to oxidative stress, were significantly induced in RBs, indicating an active metabolism of *E. ruminantium* inside host cells (for bacterial growth inside vacuoles) and the need to protect themselves against host cell defence mechanisms. Finally, the authors demonstrate that the transcription factor *dksA,* known to induce virulence in other microorganisms, is overexpressed in the infectious form of *E. ruminantium*.

In a complementary way, proteomics provides information on the protein content of cells and tissues that may differ from results at the transcriptomics level and may be relevant either for basic biological studies or for vaccine antigen discovery (Popara et al. 2015). Marcelino and co-workers used bidimensional electrophoresis (2DE) coupled to mass spectrometry (MS) analysis to establish the first 2DE proteome map of *E. ruminantium* cultivated in endothelial cells (Marcelino et al. 2012a). In 2015, the authors combined gel-based and gel-free approaches to identify proteins and/or mechanisms involved in *E. ruminantium* virulence, by performing an exhaustive comparative proteomic analysis between a virulent strain (ERGvir) and its high-passaged attenuated strain (ERGatt). Despite their different behaviours in vivo and in vitro, the results from 1DE-nanoLC-MS/MS showed that the two strains share 80 % of their proteins; this core proteome includes chaperones, proteins involved in metabolism, protein–DNA–RNA biosynthesis and processing, and bacterial effectors. Conventional 2DE revealed that 85 % of the identified proteins are proteoforms, suggesting that post-translational modifications (namely glycosyl-

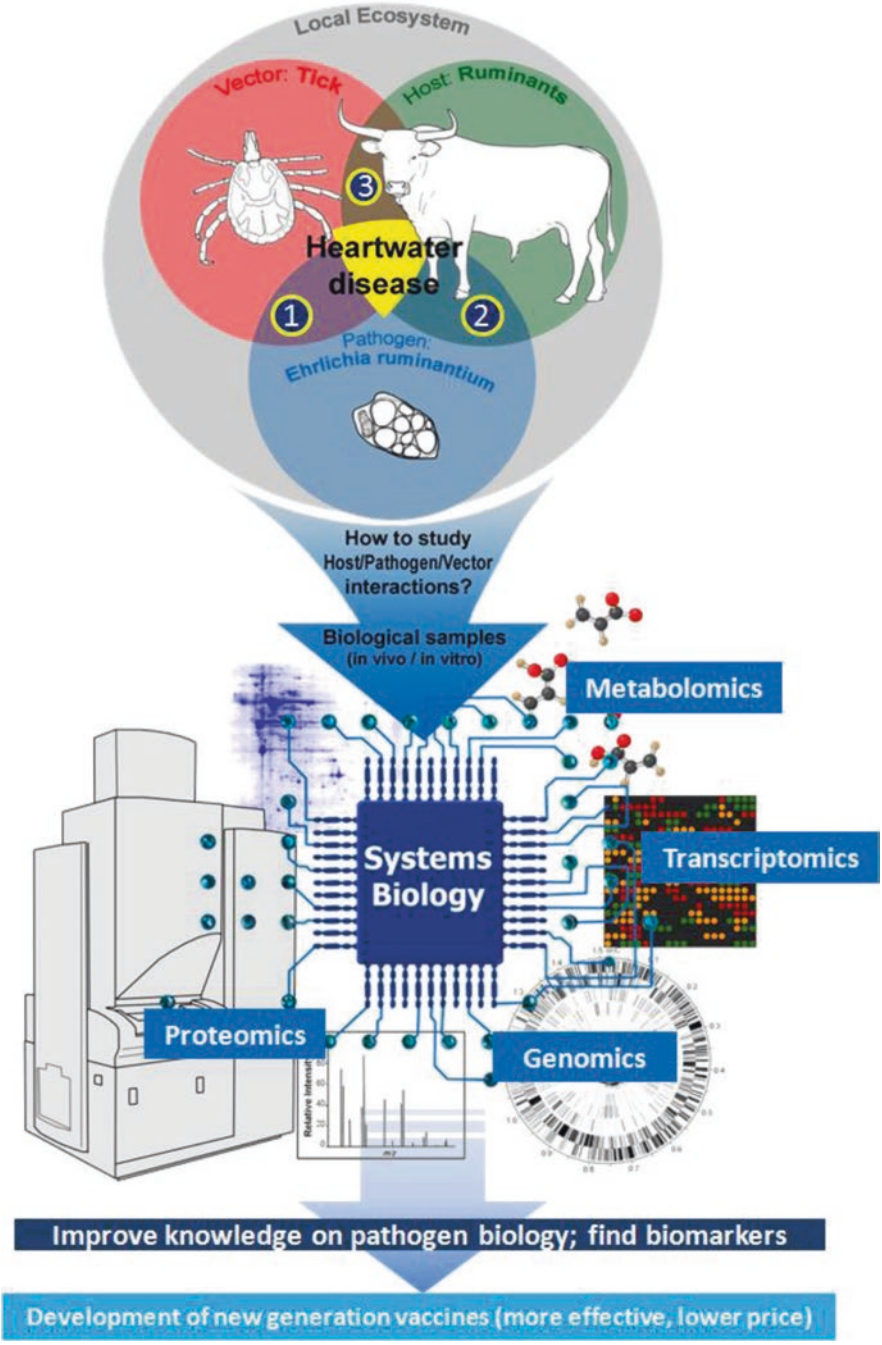

Fig. 13.10 The triangle of interactions between tick-borne pathogen, their vector and vertebrate host. The development of new vaccines against tick-borne diseases such as heartwater requires the profound knowledge of the intimate relations between (1) pathogen–tick, (2) pathogen–host and (3) tick–host (Marcelino et al. 2012b)

ation) are important in *E. ruminantium* biology. Strain-specific proteins were also identified: while ERGatt has an increased number and overexpression of proteins involved in cell division, metabolism, transport and protein processing, ERGvir shows an overexpression of proteins and proteoforms (DIGE experiments) involved in pathogenesis such as Lpd, AnkA, VirB9 and B10, providing molecular evidence for its increased virulence in vivo and in vitro. Overall, this work revealed that ERGvir and ERGatt proteomes are streamlined to fulfil their biological function (maximum virulence for ERGvir and replicative capacity for ERGatt), and the authors provide both pioneering data and novel insights into the pathogenesis of this obligate intracellular bacterium (Marcelino et al. 2015b). This work also suggests that virulence or attenuation phenomena might not be only a question of presence or lack of a specific protein but can also result from differential levels of expression of common proteins. Bioinformatics tools can also be very useful to identify virulence factors, in particular, those secreted by the Type Four Secretion System (T4SS). In 2013, Meyer and co-workers developed an algorithm to search for putative Type Four Effectors (T4Es) in the whole genome of *E. ruminantium*. This tool called S4TE (searching algorithm for type-IV secretion system effectors) predicts and ranks T4E candidates by using a combination of 13 sequence characteristics, including homology to known effectors, homology to eukaryotic domains, presence of subcellular localization signals or secretion signals, etc. Recently, Tago and Meyer (2016) used game theory tools to provide a theoretical basis to the process of generating attenuated strains of obligate intracellular bacterial pathogens. Interestingly, the authors hypothesize that *E.ruminantium* virulence might not just be a reflect of the bacterium's characteristics but would be the outcome of the interaction between the bacterium and the host defense system.

"Omics" approaches have been also very useful to study tick saliva. This fluid contains a cocktail of, potent anti-haemostatic, anti-inflammatory and immunomodulatory molecules, and it became in the last 10 years an attractive target to control tick-borne diseases. Sialome (or saliva transcriptome/proteome) studies benefit from recent advances in molecular biology, protein chemistry and computational biology, and highlighted newly isolated genes that code for proteins with homologies to known proteins allowing identification or prediction of their function. However, most of these genes code for proteins with unknown functions therefore opening new ways to functional genomic approaches to identify their biological activities and roles both in blood feeding and pathogen transmission (Valenzuela 2004). A recent proteomics study on *Amblyomma* spp., revealed that host and tick proteins involved in blood digestion, heme detoxification, development and innate immunity were differentially represented between adults and nymphs, whereas proteins involved in tick attachment, feeding, heat shock response, protease inhibition and heme detoxification were differentially represented between *Amblyomma* spp., suggesting adaptation processes to biotic and abiotic factors (Villar et al. 2014).

9 Conclusions and Future Perspectives

The vaccination strategies developed so far have proven not to be fully effective due to genetic and antigenic diversity of *E. ruminantium*. At the moment, the experimental inactivated vaccine is the most suitable for large-scale application, because of the availability of an optimized industrial process and the ability to include several strains within the vaccine to design an appropriate regional vaccine. To improve the vaccine efficacy, it will be necessary to isolate in vitro several strains from each enzootic geographic region to study their ability of protection; genotyping of protective strains will be also crucial to identify genetic markers linked to clusters of protection. More globally, it is essential to perform molecular epidemiology studies to evaluate the variability of strains in order to design regional vaccines.

On the other hand, further studies are required to better understand *E. ruminantium* pathogenesis in order to identify protective antigens and elaborate next generation vaccines. New breakthroughs in vaccine research are increasingly reliant on novel "Omics" approaches such as genomics, proteomics, transcriptomics, and other less known "Omics" such as metabolomics, immunomics, and vaccinomics (Bagnoli et al. 2011). These "Omics" approaches will deepen our understanding on: (1) *E. ruminantium* pathogenesis and attenuation mechanisms, (2) *E. ruminantium* host subversion mechanisms (including those driven by tick saliva), and (3) the key biological processes leading to protective immunity. These high-throughput technologies will also significantly contribute to overcome knowledge gaps on the role of key parasite molecules involved in cell invasion, adhesion, tick transmission and, surely revolutionize the capacity for discovering potential candidate vaccines, such as proteins involved in protective immune response, tick feeding or parasite development. These studies will contribute to the development of new treatments or next-generation vaccines.

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Part V
Midichloria

Chapter 14
Transmission of Members of the "*Candidatus* Midichloriaceae" Family to Vertebrates and Possible Involvement in Disease Pathogenesis

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1 General Considerations of "*Candidatus* Midichloriaceae" Potential Pathogenicity

"*Candidatus* Midichloriaceae", a new family of the order *Rickettsiales*, was discovered only recently thanks to molecular characterization techniques. The first report of a "*Candidatus* Midichloriaceae" endosymbiont of *Acanthamoeba* was in 1999 (Fritsche et al. 1999), and formal taxonomic recognition as a family within *Rickettsiales* was proposed in recent years (Montagna et al. 2013). Hereafter, the taxonomic category name *Candidatus* will be abbreviated to *Ca.* (for the explanation on its meaning see Chap. 3: "Biodiversity of non-model *Rickettsiales* and their association with aquatic organisms").

As shown in detail in Chap. 3, "*Ca.* Midichloriaceae" display a variability both in terms of molecular divergence and of host spectrum at least comparable to the two widely investigated families of *Rickettsiales*, namely *Rickettsiaceae* and *Anaplasmataceae*, which are responsible for several pathological alterations in humans and animals (Dumler and Walker 2005). Moreover, several "*Ca.* Midichloriaceae" bacteria have been retrieved in association with pathogenic amoebae and ectoparasites (i.e. ticks, fleas), suggesting that these bacteria could also potentially be infectious to vertebrates and even responsible for human and animal diseases However, compared with *Rickettsiaceae* and *Anaplasmataceae*,

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the mechanisms of interaction between "*Ca*. Midichloriaceae" and eukaryotic hosts, and their ability to infect different host species, are still poorly studied.

Few studies on the interaction between midichloriaceae and their hosts included investigations of the tick *Ixodes ricinus* and its endosymbiont "*Ca*. Midichloria mitochondrii" (e.g. Sassera et al. 2011), and *Acanthamoeba* infected with "*Ca*. Jidaibacter acanthamoeba" (Schulz et al. 2016) (see Chap. 3 for details). Moreover, although the phylogenetic analysis of "*Ca.* Midichloriaceae" associated with different eukaryotic hosts clearly indicates that they were repeatedly able to move and become adapted from one host to another, this aspect was rarely investigated experimentally and only included protist hosts (Schulz et al. 2016; Senra et al. 2016).

Despite the limited number of dedicated studies, some convincing indications of potential pathogenicity to humans and other vertebrates are already available for representatives of "*Ca*. Midichloriaceae". At least three cases have been documented that clearly suggest some linkage between pathogenic or immunogenic effects in vertebrates and bacteria belonging to "*Ca*. Midichloriaceae", two of which involve humans:

- "*Ca*. Midichloria mitochondrii" or strictly related bacteria can be transmitted after tick bite in humans and other mammals;
- "*Ca*. Lariskella arthropodarum" can be transmitted after tick bite in humans, which manifest acute fever symptoms;
- The presence of a "*Ca*. Midichloria"-related organism is associated with pathogenic effects in the rainbow trout *Oncorhynchus mykiss*.

The pathological implications of "*Ca*. Midichloriaceae" bacteria will be the subject of this chapter.

2 Transmission of "*Ca*. Midichloria mitochondrii" and Strictly Related Bacteria to Vertebrates

The mechanisms of interaction and the relationship of "*Ca*. M. mitochondrii" with its tick host *I. ricinus* still need to be fully elucidated (Fig. 14.1) (see Chap. 3 for details). On the other hand, this bacterium has recently attracted attention because of direct and indirect evidence for a potential pathogenic role in humans and other vertebrates (Mariconti et al. 2012a; Bazzocchi et al. 2013).

In the "*Ca*. M. mitochondrii" genome a set of 26 flagellar genes was found, including components of hook, filament and basal body, which putatively encode for functional flagella (Sassera et al. 2011). Although complete flagella have never been observed by electronic microscopy, the expression of a subset of the flagellar genes was demonstrated at the RNA level at several stages of *I. ricinus* development. In particular, all flagellar genes of "*Ca*. M. mitochondrii" were simultaneously expressed in *I. ricinus* eggs and female adults (Mariconti et al. 2012b).

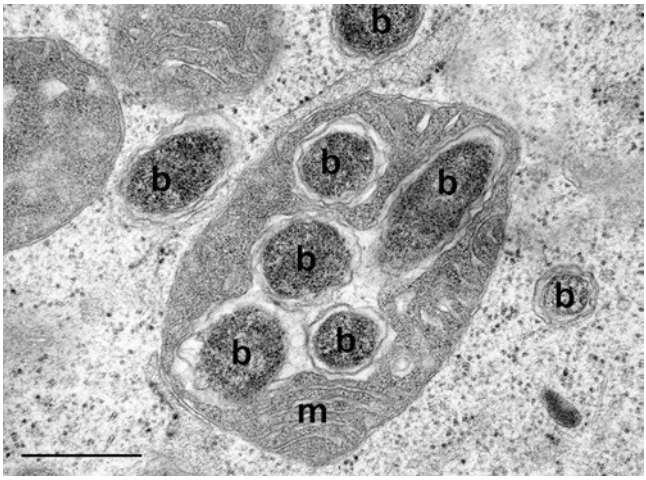

Fig. 14.1 "*Candidatus* Midichloria mitochondrii" bacteria in an oocyte of the tick *Ixodes ricinus*. *b* indicates the bacteria, *m* indicates a mitochondrion. A group of five bacteria is clearly visible within one organelle, while other "*Ca*. M. mitochondrii" appear to be in the cytoplasm, engulfed by a host-derived membrane. Bar = 0.7 μm

By applying an immunofluorescence technique using polyclonal antibodies against a fragment of the flagellar protein FliD of "Ca. M. mitochondrii", it became evident that "Ca. M. mitochondrii" is present both in ovaries and in salivary glands of the adult female tick I. ricinus. The presence of bacteria in salivary glands was also confirmed by PCR (Mariconti et al. 2012a). Such findings led to the hypothesis of transmission of "Ca. M. mitochondrii" bacteria to humans or animals by means of the tick bite, as occurs with other tick-borne bacteria such as Borrelia, Anaplasma and Rickettsia (Pesquera et al. 2015).

Serological analysis, using the recombinant FliD protein as antigen, confirmed the presence of an immunological response in humans and dogs after the tick bite (Mariconti et al. 2012a; Bazzocchi et al. 2013). Moreover, circulating DNA of "*Ca*. M. mitochondrii" and related bacteria has been found in the blood of sheep, horses, dogs and roe deer exposed to ticks (Skarphédinsson et al. 2005; Bazzocchi et al. 2013).

Taken together, these data clearly indicate that "*Ca*. M. mitochondrii" can be considered at least as a "package of antigens and DNA" that is transmitted to vertebrates through bite of its tick host. It is not clarified, however, whether the transmission involves live bacterial cells, nor if they are actually able to multiply within the vertebrate host. Nevertheless, according to the authors' interpretation, replication in the vertebrate is probable, as the small initial quantity of bacterial cells (or cell components) transferred during the tick bite would have been most likely in sufficient for detection, especially in large animals such as horses (Bazzocchi et al. 2013).

3 Transmission of "*Ca*. Lariskella arthropodarum" to Vertebrates

"*Ca*. Lariskella arthropodarum" is another member of the "*Ca*. Midichloriaceae" for which at least transient transmission to humans was observed. Mediannikov and co-authors (2004) reported that some patients affected by an acute febrile disease after a tick bite, presented, in their blood or near the area of tick bite, a 16S rRNA gene sequence corresponding to a midichloriacea retrieved in association with ticks collected in the same area (District of Khorovsk, East Russia). The authors called this organism "Montezuma", and considered it the suspected etiological agent of the disease. Later, this bacterium was found to be evolutionarily closely related to endosymbionts of other arthropods, such as stinkbugs and fleas, which were all subsequently included in the new species "*Ca*. Lariskella arthropodarum" (Matsuura et al. 2012).

According to the description by the authors, the reported symptoms of the disease resembled those of rickettsiosis and anaplasmosis, namely respiratory disorders, left shift of leukocyte formula, increased erythrocyte sedimentation rates, and increased level of serum transaminases (Mediannikov et al. 2004). However, PCR tests did not detect DNA of the etiological agents of monocytic and granulocytic ehrlichiosis, tick-borne rickettsiosis, nor other tick-borne transmissible diseases such as Lyme disease and babesiosis. On the other hand, while "*Ca*. Lariskella arthropodarum" DNA was found only in 4 out of the 22 patients examined, this could be due to assay detection limits (for example, in one patient it was found in a biopsy of the area of tick bite but not in the blood).

Nevertheless, it was not possible to establish a direct causal link of the organism to the disease although the available data are in part comparable to those on "*Ca*. Midichloria mitochondrii", as evidence of transmission of molecules to humans from "*Ca*. Midichloriaceae" bacteria after tick bites. On one hand, the case of "*Ca*. Lariskella arthropodarum" is even more relevant, because disease symptoms were also found but, on the other hand, the interaction with the vertebrate was less definite, since no specific test for host immune response against "*Ca*. Lariskella arthropodarum" was possible.

4 "*Ca*. Midichloria"-Like Organism in Rainbow Trout, *Oncorhynchus mykiss* Walbaum, Affected by Red Mark Syndrome

Red mark syndrome (RMS) is a non-debilitating condition affecting farm-reared rainbow trout (Fig. 14.2). It is characterized by the presence of red, pleomorphic, slightly raised lesions, typically on the flanks of affected fish, and generally occurs in fish > 100g. Although the disease does not cause mortality and fish can recover completely, with full healing of the lesions, the morbidity level can reach up to 90 %

Fig. 14.2 Rainbow trout (*Oncorhynchus mykiss* Walbaum) with lesions of red mark syndrome (RMS)

of an affected stock (Verner-Jeffreys et al. 2008). Fish behavior and appetite appear unaffected by the disease, but the lesions are unsightly and the negative economic impact can be severe if an outbreak occurs in stocks which are of market size, through downgrading of affected fish.

The condition was first recognized in Idaho, USA, during the 1950s, and by the 1970s was endemic in some hatcheries in the western and Pacific north-western regions (Erickson 1969; Olson et al. 1985). In the USA, the disease is referred to as strawberry disease (SD) which reflects the bright red coloration of the lesions resulting from dilation and congestion of the microvasculature (Olson et al. 1985). In 2003, a disease of rainbow trout, with lesions resembling those of SD, was detected in Scotland, UK (Noguera et al. 2007). This disease, referred to as RMS, had spread to rainbow trout farms across the UK by 2007 (Verner-Jeffreys et al. 2008). The occurrence of RMS at colder water temperatures (usually below 16 °C) and similarities in clinical signs suggested that SD from the USA and RMS were the same (Ferguson et al. 2006; Verner-Jeffreys et al. 2008). This has now been verified by pathologists from Europe and the USA, based on the case definitions of the two diseases (Oidtmann et al. 2013). RMS has also been reported from Switzerland, Austria, Italy, Turkey, Chile, and Iran (Schmidt-Posthaus et al. 2009; Galeotti et al. 2011; Kubilay et al. 2014; Sandoval et al. 2016; Sasani et al. 2016).

RMS is characterized at the histological level by dermal infiltration of lymphocytes and mononuclear cells, causing deformation and swelling of the laminar collagen structure of the stratum compactum. Edema of the scale pocket and osteoclastic resorption of the scales can be observed. Infiltration into the subcutaneous adipose tissue and skeletal muscle becomes more severe. In the initial stages, there is no involvement of the epidermis but, as the lesion develops, inflammation can extend into the epidermis and detachment of the epidermis may follow in advanced lesions, probably due to mechanical abrasion. However, it is clear that the lesion develops "from the inside out". A histological investigation of RMS lesion development in July, at a water temperature of 15 °C, suggested that the initial host cellular response is targeted toward the stratum spongiosum, with lymphocyte accumulation immediately below and above the scale pocket (McCarthy et al. 2013). A slight infiltration to the region surrounding the scale pocket was detected also in apparently healthy skin from affected trout. This observation from RMS-affected trout in Scotland is consistent with the report from SD-affected fish in the USA by Lloyd

et al. (2008), of inflammation beginning in the stratum spongiosum, which was also observed in healthy skin from affected fish and in some cohabitating unaffected fish. During the healing process, scales are regenerated; at the gross level, this is visible as a flat, grayish mark with no swelling, redness, or excess mucus. Once the fish is recovered, it is thought that there is no recurrence of the lesions in that individual (Olson et al. 1985; McCarthy et al. 2013).

Despite evidence from epidemiological studies and cohabitation trials that RMS is transmissible, numerous investigations have failed to uncover or confirm an etiological agent (Erickson 1969; Olson et al. 1985; Verner-Jeffreys et al. 2008). *Flavobacterium psychrophilum* has been suggested as one possible candidate (Ferguson et al. 2006). Using a nested PCR assay, Lloyd et al. (2008) reported a statistically significant association between SD lesions and the presence of 16S rDNA sequence similar to members of the order *Rickettsiales*. Phylogenetic analysis of the *Rickettsia*-like organism (RLO) 16S rDNA suggested that the most closely related organism was "*Ca.* M. mitochondrii", isolated from ixodid ticks. This relationship was confirmed by Montagna et al. (2013). Using the same nested PCR assay, a product of identical sequence has been amplified from rainbow trout affected by RMS in the UK (Metselaar et al. 2010; McCarthy unpublished). Subsequently, Cafiso et al. (2015) provided quantitative molecular evidence for the presence of the *Midichloria*-like organism in the organs (heart, liver, spleen, intestine, kidney) as well as the skin of RMS-affected fish.

Despite the strong statistical association with RMS, the RLO has not been confirmed as the etiological agent, and many questions remain to be answered about disease mechanisms and routes of transmission. The pathogenesis has yet to be elucidated; it has been postulated that RMS may represent a hypersensitivity response to a subclinical bacterial infection, in combination with precipitating risk factors (Olson et al. 1985; Noguera 2008). However, the belief that the manifestation of RMS lesions in an individual fish is a single, non-recurring event does not fit with the pattern of many hypersensitivity reactions, which are usually recurring or chronic (McCarthy et al. 2013). It does appear that there may be a host immunity component to the disease, as development of lesions can sometimes be triggered by stressful handling procedures, such as grading, and while outbreaks typically last 8 weeks, healing of lesions can occur very rapidly once temperatures rise toward 16 °C. It is not known if individual fish which have recovered from an outbreak are carriers of the putative etiological agent. The principle route for introduction of the disease appears to be movement of fish or ova onto a site (Adam 2009) and RMS is known to be transmissible via fertilized eggs, although disease outbreaks have not occurred at all sites stocked with fish grown from these eggs (Verner-Jeffreys et al. 2008).

RMS has commonly been managed through the use of antibiotics, particularly oxytetracycline, though the lesions will resolve without treatment (RMS Meeting 2009; Oidtmann et al. 2013). It has sometimes been possible for larger producers to maintain a supply of marketable trout through sourcing fish from a number of different production sites, which are at different stages of the disease, or by harvesting for fillet production. In the absence of definitive information about the etiological

agent, disease triggers and routes of transmission, the utilization of good biosecurity procedures is recommended to reduce the introduction and spread of disease through a site (Adam 2009). It appears that some sites have been able to eradicate the condition through systematic clearance of livestock, and disinfection or liming of ponds (Rodger 2008; Verner-Jeffreys et al. 2008). However, the continual emergence of RMS in new countries and the persistent recurrence of the disease on production sites in the UK underline the importance of identifying the etiological agent(s), so that detection and preventive measures can be targeted more effectively.

5 Conclusions and Future Perspective

As presented above in detail, after less than 20 years from the detection of the first representatives of "*Ca*. Midichloriaceae", several lines of evidence convincingly indicate that this family includes agents transmissible to humans and other vertebrates, and possibly involved in the development of pathological changes. These data clearly underline the need for further investigations to evaluate the propagation and dissemination of these bacteria inside vertebrates and the possible pathogenic effects, considering also that a formal proof of their association is lacking even in the three reported cases.

For example, human or animal diseases suspected to be linked with ticks or other ectoparasites could be reinterpreted and further investigated, in particular those clinical cases compatible with rickettsiosis or ehrlichiosis, but where there is an absence of antibody titers for known pathogenic *Rickettsiales* (Mediannikov et al. 2004). The involvement of tick-borne "*Ca*. Midichloriaceae" bacteria in modulating the immune response after the tick bite could also have consequences for the establishment of infections by other tick-borne pathogens (Mariconti et al. 2012a).

Similarly, considering that amoebae hosting "*Ca*. Midichloriaceae" are known to parasitize humans (Fritsche et al. 1999; Schulz et al. 2016), the direct involvement of bacteria in the amoeba-induced pathogenic effects as well as their infectivity could be investigated in more detail. Indeed, the role of amoebae and other protists as natural reservoirs and potential vectors for pathogenic bacteria has already been outlined (Barker and Brown 1994; Gao et al. 1997; Molmeret et al. 2005).

Aside from the aspects related to human health, the presence of several "*Ca*. Midichloriaceae" in aquatic hosts, in particular different ciliates (Vannini et al. 2010; Boscaro et al. 2013a, b; Szokoli et al. 2016; Senra et al. 2016) also calls for further investigations. These protists could be involved as reservoirs of bacteria infectious for aquatic invertebrates and vertebrates, in particular in aquaculture. As exemplified by the case of RMS in trout, the potential impact of such infectious diseases could be relevant in terms of economic consequences.

Finally, the importance of obtaining genomic sequences from other representatives of "*Ca*. Midichloriaceae" should be emphasized, as few are currently available (see "Chap. 3" for details). As in the case of "*Ca*. M. mitochondrii", genome analysis

could help in elucidating new aspects of the biology of the bacterium potentially involved in pathogenesis, as well as providing a basis for new diagnostic tools, such as the production of specific synthetic antibodies and specific tests for immune reactivity of sera (Mariconti et al. 2012a, b; Bazzocchi et al. 2013).

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Part VI
Neorickettsia

Chapter 15
Transmission Biology, Host Associations, Distribution and Molecular Diagnostics of *Neorickettsia*

Vasyl V. Tkach and Stephen E. Greiman

1 Introduction

It is well known that blood-sucking arthropods serve as vectors of numerous viral, bacterial, and protozoal infectious diseases. It is much less known, however, that parasitic worms may also transmit disease agents, including bacteria such as *Neorickettsia* transmitted by digeneans (parasitic flukes). Transmission of two bacterial diseases, "salmon dog poisoning" in North America and Sennetsu fever in Japan, was discovered almost simultaneously during the mid-1950s (Philip et al. 1953; Philip 1955; Fukuda et al. 1954; Misao and Kobayashi 1954). A new genus, *Neorickettsia*, was established for these pathogens.

The genus *Neorickettsia* includes a small number of named species and genetic lineages of obligate intracellular bacteria normally endosymbiotic within all stages of digenean complex life cycles, where they are maintained through vertical transmission. Digenean life cycle stages parasitize different host species including a mollusk of some sort as the first intermediate host, various invertebrates and vertebrates as second intermediate hosts (a few digenean groups do not have a second intermediate host), and representatives of all classes of vertebrates as the definitive host. Under certain circumstances, neorickettsiae may be transmitted horizontally from digeneans to their vertebrate definitive hosts where they invade and multiply within various cells types. Inside the host cells neorickettsiae are found in membrane-bound vacuoles and frequently arranged in groups called morulae (Fig. 15.1). In some vertebrates, neorickettsial infections of macrophages, monocytes, and other cells result in severe, sometimes fatal, disease. Thus, *Neorickettsia* transmission systems are inextricably intertwined with complex multihost life

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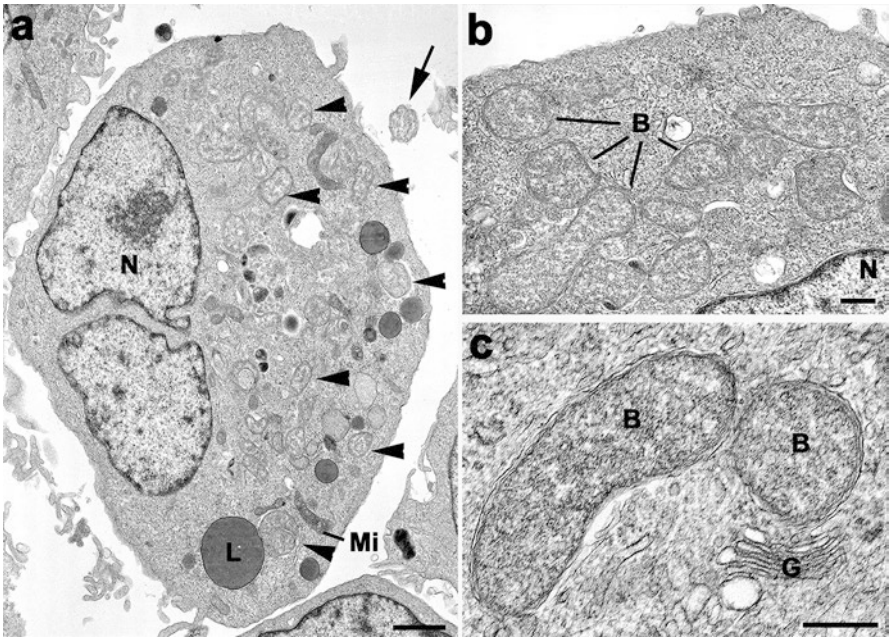

Fig. 15.1 Electron microscopic appearance of *N. sennetsu* (ATCC VR-367, Miyayama strain) in DH82 canine monocyte cultures. (**a**) Low power micrograph of an infected cell in which a number of bacteria can be identified in the cytoplasm (*arrowheads*) in addition to the nucleus (*N*), mitochondria (*Mi*), and lipid droplet (*L*). Note the single extracellular bacterium (*arrow*). Bar equals 1 μm. (**b**) Enlargement of part of the cytoplasm showing a number of gram negative bacteria (*B*). *N* nucleus. Bar equals 200 nm. (**c**) Detail of *N. sennetsu* (*arrow*) showing the gram negative bacteria limited by two unit membranes located within a membrane bound vacuole. *G* Golgi stack. Bar equals 200 nm (from Dittrich et al. 2015)

cycles of digeneans. Several previous significant reviews covered different aspects of *Neorickettsia* and neorickettsial diseases (Rikihisa 1991, 2003, 2010; Mulville 1991; Palmer 1993; Walker and Dumler 1996; Madigan and Pusterla 2000; Headley et al. 2011; Vaughan et al. 2012).

Most of the advances in our knowledge of the biology, host associations, and distribution of *Neorickettsia* in the last 20 years are linked to the development of molecular approaches and techniques, particularly PCR and DNA sequencing. Although the 16S rRNA region remains the primary target for both diagnostics and phylogenetic analyses of *Neorickettsia*, several other genes have been proposed and used for both diagnostics and phylogenetics (see Vaughan et al. 2012; Greiman et al. 2014; Greiman et al. in press). Complete genomes of four *Neorickettsia* species have recently been published, namely, *N. sennetsu* (Hotopp et al. 2006), *N. risticii* (Lin et al. 2009), *N. helminthoeca* (Lin et al. unpublished; GenBank NZ_CP007481), and *Neorickettsia* sp. (Mitreva et al. unpublished; GenBank LNGI01000001). This allows for quick progress in our understanding of the biology, biochemical, and

immunological properties of these bacteria using genomic tools (Lin et al. 2009). Neorickettsiae have the smallest genome size of all members of the Family Anaplasmataceae; for instance, the genome of *N. risticii* consists of only 879,977 nucleotide base pairs. Genome-wide comparison between *N. risticii* and *N. sennetsu* showed that very high percentages (75 % or 88.2 %) of protein-coding genes are conserved between these *Neorickettsia* species. Moreover, comparison of genes among *N. risticii* and other Anaplasmataceae showed that most genes are shared among other members of major Anaplasmataceae lineages. Genomic analysis allowed for the identification of certain genes potentially involved in the pathogenesis of *N. risticii* (Lin et al. 2009) which opens new avenues for vaccine development.

2 Systematics and Phylogeny of *Neorickettsia*

2.1 Position of Neorickettsia *Among Rickettsiales*

The current classification of rickettsiae and related intracellular bacteria is mostly based on molecular data. According to the results of a molecular phylogenetic analysis, primarily based on the 16S rRNA and *groESL* gene sequences, the order Rickettsiales is now split into two families, Rickettsiaceae and Anaplasmataceae (Dumler et al. 2001). Other characters used to justify this systematic arrangement, included outer membrane protein sequences and biological characteristics such as morphology, host cell tropism, disease ecology, and clinical picture. *Neorickettsia* is one of four generally accepted genera belonging to the family Anaplasmataceae, namely, *Anaplasma*, *Ehrlichia*, *Wolbachia*, and *Neorickettsia* (Dumler et al. 2001; Tallardat-Bisch et al. 2003; Rikihisa 2010; Fig. 15.2). Recent publications (Seng et al. 2009; Rikihisa 2010; Greiman et al. 2014) may suggest the existence of at least one additional genus which has not been sufficiently characterized beyond obtaining sequence data and thus has not been formally described yet. *Neorickettsia* is the most genetically divergent genus of the Anaplasmataceae. It is also unique among other genera due to the presence of both vertical transmission and horizontal transmission (Cordy and Gorham 1950; Gibson et al. 2005). *Neorickettsia* can be grown in vitro within certain cell types (e.g., macrophages and monocytes) of various vertebrate species (e.g., mouse, dog, human).

2.2 Phylogenetic Interrelationships Within Neorickettsia

Presently, there are four formally named species and 20 species-level genetic lineages of *Neorickettsia* (Table 15.1, Fig. 15.3), five of which cause distinct diseases as described later. The genetic differentiation between species in the literature is based primarily on 16S rRNA sequence divergence levels (Dumler et al. 2001; Stackebrandt et al. 2002). Biological differences among neorickettsiae are seen at

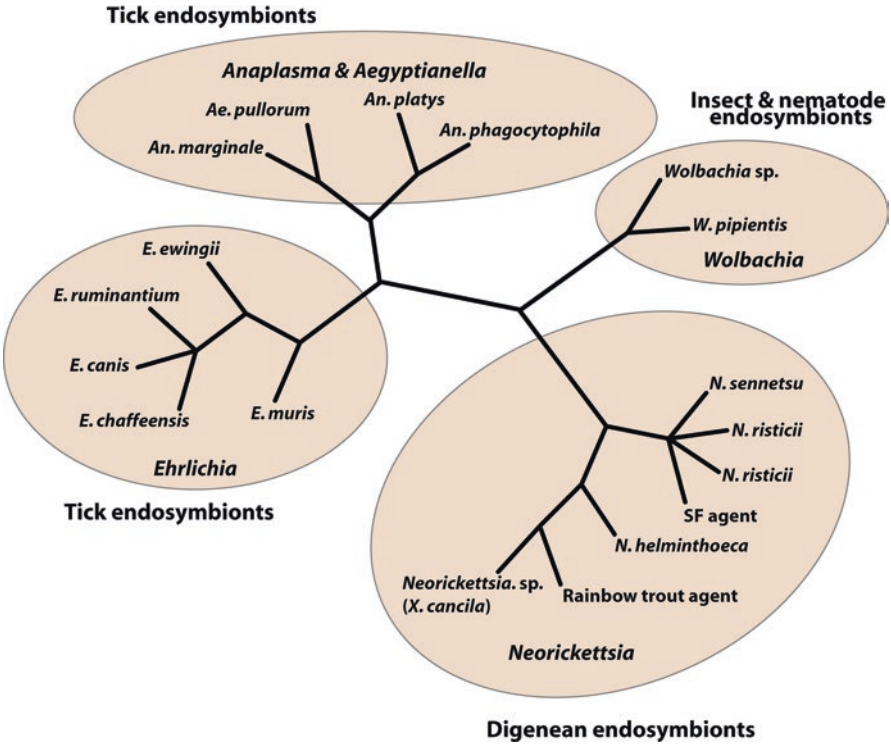

Fig. 15.2 Phylogenetic interrelationships among the genera of Anaplasmataceae based on Bayesian analysis of partial 16S rRNA sequences. Vector hosts are indicated next to bacterial genera. Not all known genetic lineages of *Neorickettsia* are included (from Vaughan et al. 2012)

the level of host specificity and pathogenicity to different hosts (antigenic properties, etc.). It should be mentioned that some neorickettsial species demonstrate immunological cross-protection and corresponding serological cross-reactivity. For instance, *N. sennetsu* causes illness in humans but is not known to cause disease in horses. However, upon experimental exposure to *N. sennetsu*, horses become immune against *N. risticii*, a species pathogenic to horses (Rikihisa and Jiang 1988). Similarly, *N. elokominica* is quite pathogenic to black bears but causes only mild clinical manifestations in dogs. When dogs are inoculated with *N. elokominica* they acquire immunity to *N. helminthoeca*, which is normally highly virulent to naïve dogs (Farrell et al. 1973).

Currently, the criteria for species differentiation among *Neorickettsia* are not well established. The relatively well-studied species causing animal and human diseases, namely, *N. helminthoeca*, *N. risticii*, and *N. sennetsu*, are well characterized genetically and clinically, but the level of knowledge of their biology varies greatly from rather well known for the former two species to a complete lack of knowledge for the latter one. These three species have also been maintained in laboratory cell cultures.

Table 15.1 *Neorickettsia* species/genotypes, digenean family, genus and species, group of vertebrate definitive hosts and country

Neorickettsia species	Digenean family	Digenean genus and species	Vertebrate hosts	Country	Reference
Neorickettsia helminthoeca	Troglotrematidae	*Nanophyetus salmincola*	Fishes and mammals	United States	Philip et al. (1953)
Neorickettsia elokominica	Troglotrematidae	*Nanophyetus salmincola*	Fishes and mammals	United States	Farrell et al. (1973)
Neorickettsia risticii	Dicrocoeliidae	*Conspicuum* sp.	Birds	United States	Tkach et al. (2012)
	Echinostomatidae	*Echinoparyphium rubrum*	Birds	United States	Tkach et al. (2012)
	Echinostomatidae	*Echinostoma cinetorchis*	Mammals	South Korea	Park et al. (2003)
	Echinostomatidae	*Echinostoma hortense*	Birds and mammals	South Korea	Park et al. (2003)
	Fasciolidae	*Fasciola* sp.	Mammals	South Korea	Park et al. (2003)
	Heronimidae	*Heronimus mollis*	Turtles	United States	Tkach et al. (2012)
	Lecithodendriidae	*Lecithodendrium* sp.	Mammals and birds	United States	Pusterla et al. (2003)
	Lecithodendriidae	*Acanthatrium* sp.	Mammals	United States	Pusterla et al. (2003)
	Lecithodendriidae	*Acanthatrium oregonense*	Mammals	United States	Gibson et al. (2005)
	Macroderoididae	*Alloglossidium corti*	Fishes	United States	Tkach et al. (2012)
	Microphallidae	Microphallidae sp.	Bird or mammal	South Korea	Park et al. (2003)
	Schistosomatidae	Schistosomatidae sp.	Birds and mammals	South Korea	Park et al. (2003)
	Unknown	Unknown	Mammals	Argentina	Cicuttin et al. (2013)
	Derogenidae	*Deropegus aspina*	Fishes and amphibians	United States	Greiman et al. (2014)
	Lecithodendriidae	*Prosthodendrium* sp.	Mammals and birds	United States	Greiman et al. (2014)

(continued)

Table 15.1 (continued)

Neorickettsia species	Digenean family	Digenean genus and species	Vertebrate hosts	Country	Reference
Neorickettsia sennetsu	Unknown	Unknown	Unknown	Japan, Malaysia, Laos, Thailand	Fukuda et al. (1954); Misao and Kobayashi (1954)
Neorickettsia sp. (SF agent)	Heterophyidae	*Stellantchasmus falcatus*	Mammals	Japan, Vietnam	Fukuda and Yamamoto, (1981); Greiman et al. in press
	Heterophyidae	*Metagonimoides oregonensis*	Mammals	United States	Greiman et al. (2014)
	Troglotrematidae	*Nanophyetus salmincola*	Mammals and Fishes	United States	Greiman et al. (2016)
Neorickettsia sp. (Catfish agent#1)	Gorgoderidae	*Phyllodistomum lacustri*	Fishes	United States	Tkach et al. (2012)
Neorickettsia sp. (Catfish agent#2)	Allocreadiidae	*Megalogonia ictaluri*	Fishes	United States	Tkach et al. (2012)
Neorickettsia sp.	Unknown	Unknown	Fishes	Cambodia	Seng et al. (2009)
Neorickettsia sp.	Unknown	Unknown	Fishes	Antarctica	Ward et al. (2009)
Neorickettsia sp. (Rainbow trout agent)	Derogenidae	*Deropegus* sp.	Fishes	United States	Pusterla et al. (2000b)
	Allocreadiidae	*Crepidostomum* sp.	Fishes	United States	Pusterla et al. (2000b)
	Allocreadiidae	*Creptotrema* sp.	Fishes	United States	Pusterla et al. (2000b)
	Sanguinicolidae	*Sanguinicola* sp.	Fishes	United States	Pusterla et al. (2000b)
Neorickettsia sp. 1	Allocreadiidae	*Crepidostomum affine*	Fishes	United States	Greiman et al. (2014)
Neorickettsia sp. 2	Haploporidae	*Saccocoelioides beauforti*	Fishes	United States	Greiman et al. (2014)
Neorickettsia sp. 3	Pleurogenidae	Unknown	Mammals and amphibians	United States	Greiman et al. (2014)
Neorickettsia sp. 4	Haploporidae	*Saccocoelioides lizae*	Fishes	China, Thailand	Greiman et al. (2014); Greiman et al. in press

Neorickettsia sp. 5	Faustulidae	*Bacciger sprenti*	Fishes	Australia	Greiman et al. (2014)
Neorickettsia sp. 6 (PE agent)	Plagiorchiidae	*Plagiorchis elegans*	Birds and mammals	United States	Greiman et al. (2014)
Neorickettsia sp. 7	Diplostomidae	Unknown	Birds	United States	Greiman et al. (2014)
Neorickettsia sp. 8	Lecithodendriidae	*Lecithodendrium* sp.	Mammals (bats)	Egypt	Greiman et al. in press
Neorickettsia sp. 9	Haploporidae	*Saccocoelioides* sp.	Fishes	Vietnam	Greiman et al. in press
Neorickettsia sp. 10	Lecithodendriidae	*Paralecithodendrium* sp.	Mammals (bats)	Philippines	Greiman et al. in press
Neorickettsia sp. 11	Lecithodendriidae	*Paralecithodendrium* sp.	Mammals (bats)	Philippines	Greiman et al. in press
Neorickettsia sp. 12	Lecithodendriidae	*Lecithodendrium* sp.	Mammals (bats)	Thailand	Greiman et al. in press
Neorickettsia sp. 13	Haploporidae	*Dicrogaster* sp.	Fishes	United States	Greiman et al. in press
Neorickettsia sp.	Fasciolidae	*Fasciola hepatica*	Fishes	United States	Mitreva et al. unpublished, GenBank LNGI01000001

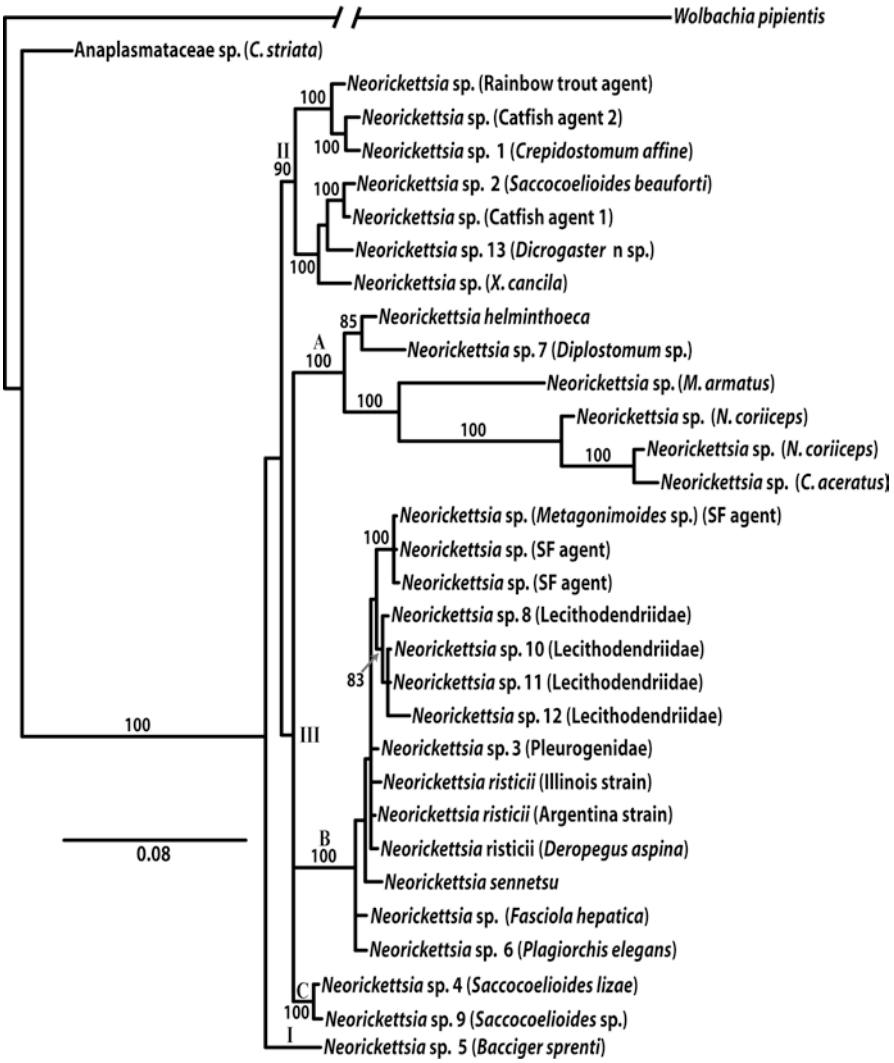

Fig. 15.3 Phylogenetic interrelationships among 29 genetic lineages of *Neorickettsia* based on Bayesian analysis of partial 16S rRNA sequences. Numbers above internodes indicate posterior probabilities

On the other hand, a fourth formally named species, *N. elokominica*, has not been sequenced or maintained in laboratory in any form other than in the infected vertebrates. The remaining 16 species-level genetic lineages of *Neorickettsia* reported in the literature remain unnamed (Vaughan et al. 2012; Tkach et al. 2012; Greiman et al. 2014; Greiman et al. in press; Table 15.1). Lack of sequence data on some of the published reports precludes proper identification of these *Neorickettsia* forms.

For instance, neorickettsiae reported from digeneans in Korea (Chae et al. 2003; Park et al. 2003) as *N. risticii* may not belong to this species. Based on the sequence divergence levels in the 16S gene provided by these authors, their isolates are more closely related to the "rainbow trout" genotype of *Neorickettsia* reported from North America. Considering that the "rainbow trout agent" and *N. risticii* are not very closely related (Fig. 15.3) the Korean samples most likely represent additional species-level lineage of *Neorickettsia*.

The molecular phylogenetic analysis incorporating all currently available 16S sequence of *Neorickettsia* shows three strongly supported clades within the genus (Fig. 15.3) similar to the topologies reported by Greiman et al. (2014) in a smaller dataset. Clade I is represented by a single sequence from *Bacciger sprenti* (Faustulidae) collected in eastern Australia. The clade II includes seven unnamed lineages/species of *Neorickettsia* found either within various digenean families that use fish as definitive hosts or in fish tissues. This clade is characterized by strongly supported, well-resolved internal branch topology.

Most *Neorickettsia* species belong to Clade III including all three currently named species of *Neorickettsia*, *N. helminthoeca*, *N. risticii*, and *N. sennetsu*. This large clade is segregated into three subclades indicated as A, B, C in Fig. 15.3. Well-supported subclade A includes a diverse assemblage of genotypes that included *N. helminthoeca*, as well as, several lineages obtained from either digenean cercariae (*Neorickettsia* sp.7) or fish tissue in Southeast Asia and Antarctica. Subclade B is 100 % supported and contains the largest number of neorickettsial genotypes, although with mostly unresolved internal topologies. This clade includes both *N. sennetsu*, causing human disease in Southeast Asia, and *N. risticii*, the causative agent of the Potomac horse fever disease. This phylogenetic affinity explains the similarities in immunological properties demonstrated by these two pathogens distributed in different parts of the world. The two *Neorickettsia* lineages in subclade C are comprised exclusively of two forms from haploporid digeneans from China and Thailand (*Neorickettsia* sp. 4) and from Vietnam (*Neorickettsia* sp. 9). Analysis of the phylogenetic interrelationships among *Neorickettsia* in connection with their host associations will be further discussed later.

3 Transmission Biology of *Neorickettsia*

There are four distinct diseases described in the medical/veterinary literature attributable to *Neorickettsia*: salmon poisoning disease (SPD), Elokomin fluke fever (EFF), Sennetsu fever, and Potomac horse fever (PHF) (Table 15.1). The agents of these diseases are described as separate species based on differing pathologies, serology, antigenic profiles and, with the exception of *N. elokominica*, DNA sequence analyses. In addition, there are at least 19 other species/genotypes of neorickettsial endosymbionts whose pathogenicity to humans or other vertebrates is not known. The discovery of *Neorickettsia* genotypes, with unknown pathologies, resulted largely from ecological studies targeting the pathogenic species. For

example, studies on Sennetsu fever discovered the SF (abbreviated from *Stellantchasmus falcatus*) agent and ecological studies on *N. risticii* discovered the neorickettsiae provisionally designated as rainbow trout agent, catfish agent 1, catfish agent 2, and undefined *N. risticii*-like agents.

3.1 Neorickettsia helminthoeca

Native Americans and white settlers along the northwestern coast of the United States knew that if dogs were allowed to eat salmon (usually dead or dying "spawned out" fish), the dogs were likely to become ill and die. It was presumed that the fish contained some toxin that was poisonous to canid mammals such as dogs, foxes, and coyotes, but not to other fish-eating carnivores, such as raccoons or mink. The disease was thus called "*salmon poisoning of dogs.*" Its characteristic symptoms were very rapid development of fever and weight loss, soon accompanied by vomiting and black, bloody diarrhea. If left untreated, the mortality rate could reach 90 % within 2 weeks after the first symptoms. However, already more than 100 years ago, Pernot (1911) demonstrated that SPD was caused by an infectious agent, not a toxin. Two to four days after blood from sick dogs was injected into healthy dogs, the injected dogs developed SPD symptoms. Serial injections of blood from these experimentally infected dogs into naïve dogs also produced SPD. It was also shown that the few dogs that survived the illness became immune to subsequent injections. The exact etiology of the infection remained unknown until Donham (1925) reported that autopsied dogs that died of SPD were all parasitized by small digenean flukes later identified as *Nanophyetus salmincola* by Chapin (1926). Donham correctly concluded that the sick dogs acquired these flukes by ingesting fluke cysts (=metacercariae) found in the flesh and internal organs of local salmonid fish and linked the disease to the presence of the fluke. This conclusion was corroborated by studies of Simms et al. (1931) who showed that both metacercariae and adult flukes of *N. salmincola* caused SPD when fed or injected into dogs. They suspected that the infection was caused by a rickettsia or hemosporidian protist. Their hypothesis was confirmed when Cordy and Gorham (1950) described intracellular rickettsial-like organisms in reticuloendothelial cells of lymph nodes from a dog that died of SPD. Philip et al. (1953) named the agent *Neorickettsia helminthoeca* in recognition that it was a new type of rickettsia and that digeneans were required for its transmission.

During the 1950s and 1960s, intensive studies of the disease and its agent at Oregon State University and the NIH Rocky Mountain Laboratory in Hamilton, MT, have demonstrated that the pathogen is present in all life stages of the digenean parasite (for detailed history and bibliography see Vaughan et al. 2012; Fig. 15.4). Interestingly, the geographic distribution of SPD is constrained by the distribution of its digenean host *N. salmincola* which is in turn determined solely by the distribution of the fluke's first intermediate host, the snail *Oxytrema silicula* (Family: Pleuroceridae).

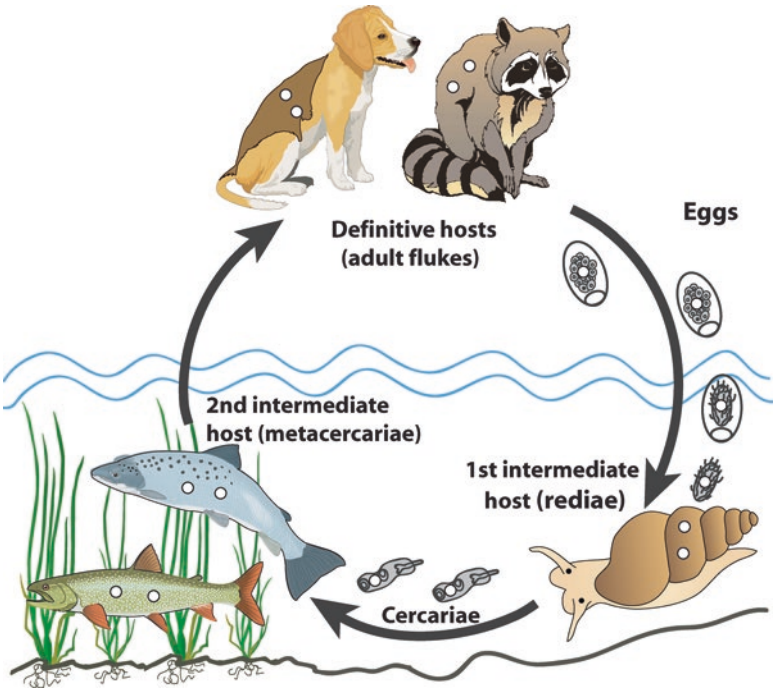

Fig. 15.4 Circulation of *Neorickettsia helminthoeca* (white dots) through life cycle of its digenean host, *Nanophyetus salmincola* (from Vaughan et al. 2012)

Dogs and other canids acquire infections by consuming fish containing metacercariae of *N. salmincola* in various tissues and organs such as muscles, kidney, and heart. Ingested metacercariae develop into adult flukes in dog's small intestine and begin producing eggs in 5–10 days, which closely corresponds to the usual prepatent period of SPD in dogs. The mechanism of horizontal transmission of neorickettsiae from the flukes to intestinal tissues and then into macrophages of the dog is still unknown. If left untreated, most dogs die within 2 weeks after the appearance of clinical symptoms. During the rapid course of disease, gross pathology is mostly restricted to the visceral lymph nodes, which become enlarged and fleshy, often with areas of hemorrhage and necrosis. Despite extremely severe consequences of infection in dogs, experimental infections of noncanid mammals, including raccoons, bears, mink, bobcats, domestic cats, guinea pigs, rats, mice, or hamsters failed to produce more than a mild fever (Cordy and Gorham 1950; Simms et al. 1932). Humans can be infected with the flukes (Eastburn et al. 1987; Harrell and Deardorff 1990) but do not develop SPD symptoms. Philip (1955) infected himself by eating raw metacercariae-infected trout, samples of which when fed to a dog resulted in a fatal infection. The researcher developed adult flukes in his intestine as confirmed by the appearance of eggs in his stool 10 days after ingestion of fish, but had no symptoms of the disease.

Salmon are normally infected with *N. salmincola* metacercariae during their years as young fish in freshwater. Farrell et al. (1964) has demonstrated that *Neorickettsiae* remain viable within *N. salmincola* metacercariae for at least 3 years during the oceanic phase of the salmon life cycle. Hence, dogs can acquire SPD by eating fingerlings, smolts, or adult fish (Millemann et al. 1964). Both the parasite and the neorickettsial endosymbiont are killed by cooking or freezing (−20 °C, 24 h). Metacercariae of *N. salmincola* may naturally infect other fishes (e.g., shiner, sculpin, lamprey) which creates the potential for canids to contract SPD beyond eating raw salmon or trout.

Until recently, SPD was considered to be restricted exclusively to the Pacific coast of the United States and Canada. In North America, SPD is only transmitted by *N. salmincola*. However, *N. helminthoeca* has been recently confirmed in south-central Brazil using immunological and molecular techniques (Headley et al. 2004, 2009). This indicates the possibility of an alternative life cycle for SPD and that it is more widespread than currently appreciated. The fluke species responsible for SPD transmission in Brazil remains unknown.

Raccoons and spotted skunks are likely the principal definitive hosts of *N. salmincola* in the Pacific northwest based on their abundance and the high prevalence (100 and 75 %, respectively) and intensities of fluke infections (average of 57,571 flukes per animal in raccoons and 2613 in skunks). Although raccoons do not become clinically ill with SPD they likely act as carriers of *N. helminthoeca* and disseminate the infection in the environment.

3.2 Neorickettsia risticii

A previously unknown illness of horses appeared in the rural counties of Maryland and Virginia in the late 1970s; symptoms usually included fever, depression, anorexia, and colitis, accompanied by acute diarrhea with frequent abortions among pregnant mares (Holland et al. 1985a; Rikihisa 1991; Coffman et al. 2008). In severe cases, horses exhibited laminitis. If left untreated, overall mortality approached 30 %. Due to the proximity of the affected area to the Potomac River the disease received the common name Potomac Horse Fever (PHF) while the clinical term coined for this condition was equine monocytic ehrlichiosis. Stools from diarrheic horses could produce PHF when fed to susceptible horses (Biswas et al. 1994). The highly seasonal occurrence of the disease (mostly summer time), combined with the fact that susceptible horses stabled with sick horses rarely acquired PHF, suggested that PHF was a vector-borne disease, rather than communicable disease.

Because the agent of the disease was initially placed in the genus *Ehrlichia* it was thought to be transmitted by ticks. However, vector competence studies failed to incriminate ticks in the transmission of the PHF (Hahn et al. 1990; Levine et al. 1990). The situation remained unresolved until Yasuko Rikihisa and her colleagues at Ohio State University used DNA sequence-based approaches to the study of ehrlichial diseases. This not only allowed for development of accurate diagnostic

tools for PHF but also provided evidence of close relationship of the PHF and Sennetsu fever agents with *N. helminthoeca*, the agent of SPD (Pretzman et al. 1995). At the time it was already known that SPD is transmitted by digeneans. Hence, it became evident that PHF circulation should involve these parasites. Soon after this discovery, *N. risticii* DNA was isolated from digenean larval stages (cercariae and sporocysts) infecting *Juga* snails inhabiting streams of an endemic area (Barlough et al. 1998; Reubel et al. 1998). When larval flukes containing *N. risticii* were inoculated into horses, the horses developed PHF. Further studies in northern California (Pusterla et al. 2000a, c, 2003; Chae et al. 2000, 2002), central Pennsylvania (Mott et al. 2002; Gibson et al. 2005; Gibson and Rikihisa 2008), and Minnesota (Farren 2007) showed that transmission cycles in these areas involve several fluke species that use snails as the first intermediate host, aquatic insects (e.g., caddisflies, mayflies) as the second intermediate host, and insectivorous birds and/or bats as the definitive hosts (Fig. 15.5). Therefore, transmission to horses supposedly occurs when they somehow swallow insects containing metacercariae. This could occur in a variety of ways—either through consuming insects while grazing, eating insect-contaminated hay, or by drinking water containing insects that have been attracted to lights at night over watering troughs and fell in the water. Thus, it appears that digenean metacercariae do not need to complete development to the adult stage in order to pass the infection to horses. Unlike the situation with SPD and dogs, horses can truly be considered "dead-end hosts" for PHF transmission.

Outside of North America, *N. risticii* has been documented using PCR techniques in Uruguay, southern Brazil, and Argentina (Dutra et al. 2001; Cicuttin et al. 2013). Indirect fluorescent antibody testing provided positive results in other regions of the world; however, it is unclear whether these could be false-positive records resulting from horse vaccination. A commercial vaccine for PHF based on the type strain, *N. risticii* Illinois, became available in the late 1980s. However, cases of vaccine failures began to appear (Vemulapalli et al. 1995), suggesting the existence of additional antigenically distinct strains of *N. risticii*. This was confirmed by the study of Chaichanasiriwithaya et al. (1994) who found three immunologically distinct *N. risticii* "sero-groups." Later, Gibson et al. (2011) reported geographic clustering among various *N. risticii* isolates collected in the USA with respect to the predicted amino acid sequences of several proteins. This supports the earlier suggestions (Barlough et al. 1998; Reubel et al. 1998) that *N. risticii* constitutes a collection of strains that may differ in the digenean hosts, snail ecology, and level of virulence to horses

3.3 Neorickettsia sennetsu

In the mid-1950s, Japanese scientists were investigating the cause of an acute rickettsial mononucleosis of humans endemic to Miyazaki Prefecture in Japan. Symptoms lasted approximately 2 weeks and included high fever, enlarged cervical lymph nodes and spleen, malaise, anorexia, and peripheral blood mononucleosis;

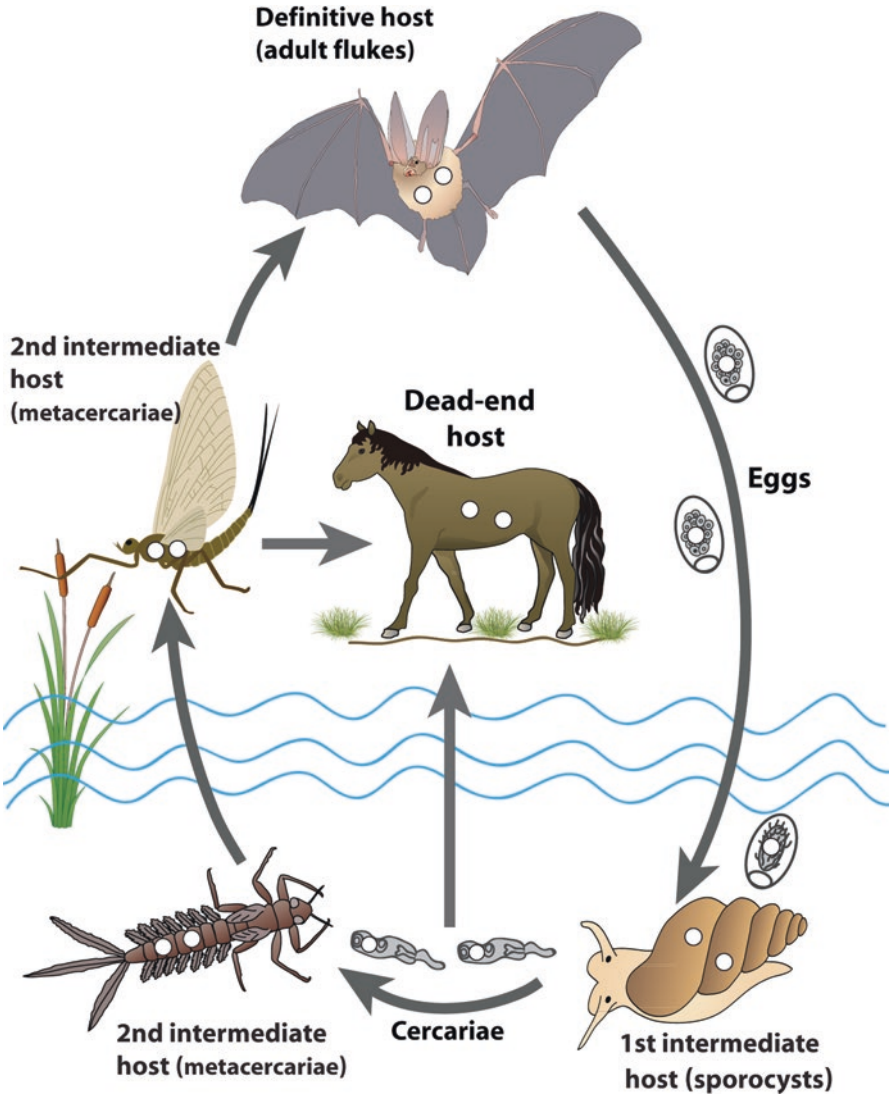

Fig. 15.5 Circulation of *Neorickettsia risticii* (white dots) involving lecithodenrdiid digeneans (e.g. *Acanthatrium* spp. in this diagram) and horses as a dead-end host (from Vaughan et al. 2012)

with no fatalities reported. Epidemiological studies identified raw grey mullet fish (*Mugil cephalus*) containing digenean metacercariae as the source of infection (Fukuda 1958) and the agent was named *Ehrlichia* (later renamed into *Neorickettsia*) *sennetsu. Neorickettsia sennetsu* was isolated from both patients and fish, but the digenean vector of this disease remains unknown. Later, *N. sennetsu* was reported from patients in Malaysia and more recently in Laos (Holland et al. 1985b;

Newton et al. 2009; Dittrich et al. 2015). In the meantime the disease has apparently disappeared from Japan. Interestingly, despite the high seroprevalence of *N. sennetsu* antibodies in the Laos population (~17 %), acute *N. sennetsu* infections are rare. Considering the high seroprevalence to *N. risticii* and the significant burden of other fish-borne diseases in the country, the very low frequency of detected *N. sennetsu* in human patients was unexpected (Dittrich et al. 2015). The previously demonstrated serological cross-reactivity among *Neorickettsia* combined with reports of multiple additional genetic lineages of *Neorickettsia* in the region (Newton et al. 2009; Seng et al. 2009; Greiman et al. 2014; Greiman et al. in press) suggests that *Neorickettsia* species other than *N. sennetsu* may be partly responsible for the high seroprevalence in human patients. This underlines the need for further investigations to elucidate if raw fish and their trematodes are indeed the mode of transmission for this pathogen (Dittrich et al. 2015).

Very little is known regarding the distribution of *N. sennetsu* among mammals other than humans, with the exception of a single report from rodents in Japan (Fukuda et al. 1962). Laboratory mammals such as monkeys, rodents, and dogs can be experimentally infected with *N. sennetsu* and develop multiple symptoms and enlarged lymph nodes (Holland et al. 1985a, b). *Neorickettsia sennetsu* can be propagated in primary canine blood monocyte cultures which provides a diagnostic and research tool (Holland et al. 1985a, b; Dittrich et al. 2015).

3.4 Neorickettsia elokominica *and* Neorickettsia *sp.* *(SF Agent)*

In the early 1970s, Farrell and colleagues published a series of papers describing what they designated as a second neorickettsial agent (in addition to SPD) transmitted by *Nanophyetus salmincola* (Farrell et al. 1973). They originally set out to determine if black bear, *Ursus americanus* (proven earlier to be refractory to SPD) could act as natural reservoir of SPD. A number of bears were fed trout infected with *N. salmincola* metacercariae. A proportion of the bears developed fever, anorexia, and 'lassitude.' Upon autopsy, the sick bears showed histological evidence of SPD. Lymph node suspensions from the autopsied bears were injected into dogs; 72 % of the injected dogs developed low-grade fever and diarrhea for 4–12 days but did not die. Three months following injection 87 % of the convalescent dogs died of SPD after being fed metacercariae-infested trout. Based on the differences in clinical symptoms and lack of conferred immunity between SPD and this disease Farrell et al. (1973) named the disease "Elokomin fluke fever" (EFF) because the source of the infective trout was the Elokomin River. Later studies, utilizing immunofluorescent antibody tests and live animal challenges, confirmed that EFF agent was different immunologically from both *N. helminthoeca* and *N. sennetsu* (Kitao et al. 1973). The agent of EFF has been provisionally designated as *Neorickettsia elokominica* in the Merck Veterinary Manual.

While searching for the digenean vector of Sennetsu fever, Fukuda et al. (1973) discovered another neorickettsial agent in metacercaraiae of the digenean *Stellantchasmus falcatus* infecting grey mullet. It has become known as "*SF agent*" and differs from *N. sennetsu* in western blot profiles, clinical and pathologic responses of mice and 16S rRNA sequence comparisons (Wen et al. 1996). SF agent is not known to cause disease in humans or in experimentally infected monkeys. The digenean *S. falcatus* has a broad geographic range (Japan, Korea, Southeast Asia, Hawaii, Australia, Egypt, Palestine and Israel according to Chai and Sohn 1988); however, SF agent (within this fluke host) has only been recorded from this fluke species from Japan and Vietnam (Fukuda et al. 1973; Greiman et al. in press). Greiman et al. (2014, in press) have also amplified and sequenced SF agent DNA from two additional digeneans, *Metagonimoides oregonensis* and *Nanophyetus salmincola*, in the United States (Table 15.1). As discussed earlier *N. salmincola* is the digenean host of *N. helminthoeca* and *N. elokominica*, the causative agents of SPD and EEF, respectively. Thus, *N. salmincola* is the only trematode species known to harbor three different species of *Neorickettsia*.

3.5 Neorickettsia *sp. Rainbow Trout Agent*

Thirty-five rainbow trout were collected by Pusterla et al. (2000b) from the Shasha River in California in an attempt to elucidate the ecology of PHF in the state. The rainbow trout were necropsied and examined for adult digeneans. Neorickettsial DNA was recovered from fish tissues and three species of flukes collected from the gall bladder and intestine of the trout (*Crepidostomum*, *Creptotrema*, *Deropegus* spp.) and from the eggs of a blood fluke (*Sanguinicola* sp.) recovered from gill capillaries (Pusterla et al. 2000b). *Neorickettsia* 16S sequences obtained from fish tissue and digeneans were identical to each other, although different enough from *N. risticii*, *N. sennetsu,* SF agent, and *N. helminthoeca* (95–96 % sequence homology) to suggest that the "rainbow trout agent" is a new genotype. Currently, nothing is known about its pathogenicity in fish or fish eating vertebrates.

3.6 Neorickettsia *sp. (PE Agent)*

Through an effort to better understand the transmission biology of *Neorickettsia* within its digenean host Greiman and colleagues screened a number of digenean cercariae from aquatic snails collected from northeastern North Dakota. As a result they identified a novel species of *Neorickettsia* from a digenean *Plagiorchis elegans* (*Plagiorchis elegans* (PE) agent) (Greiman et al. 2013). *Plagiorchis elegans* is a generalist parasite utilizing multiple groups of invertebrates as second intermediate hosts, as well as, a wide range of vertebrates as a definitive host. In North Dakota, muskrats are the most common natural definitive host (Fig. 15.6).

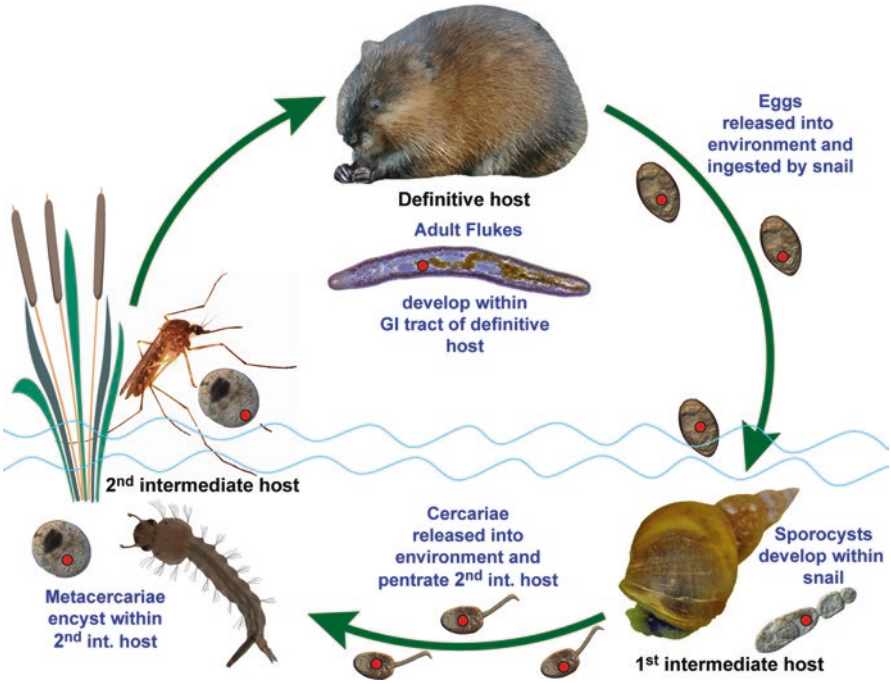

Fig. 15.6 Circulation of *Neorickettsia* sp. (red dots) through natural life cycle of the digenean *Plagiorchis elegans* (from Greiman et al. 2013)

Greiman et al. (2015) established and maintained a laboratory life cycle of *P. elegans* harboring *Neorickettsia*. The laboratory life cycle consisted of a snail first intermediate host (*Lymnaea stagnalis*), an aquatic mosquito second intermediate host (*Culex pipiens*), and a hamster definitive host (*Mesocricetus auratus*; belonging to the same family Cricetidae as muskrat) (Fig. 15.7). The maintenance of neorickettsiae throughout the complete life cycle of *P. elegans* in the laboratory allowed for controlled studies on the transmission biology of the bacteria with its digenean host. This includes the analysis of vertical transmission efficiency and localization of *Neorickettsia* within the digenean host. As a result, PE agent is currently the best studied species of *Neorickettsia* in regards to its transmission biology (Greiman et al. 2013, 2016; Greiman and Tkach 2016).

Greiman et al. (2016) used indirect immunofluorescence microscopy to study the localization of PE agent in organs and tissues within different developmental stages of its digenean host, *P. elegans*. They documented the distribution of bacteria within sporocysts, cercariae, metacercariae, and adults containing eggs obtained from the laboratory-maintained life cycle. Neorickettsiae were found within the tegument of sporocysts, throughout cercarial embryos (germ balls) and fully formed cercariae (within the sporocysts), throughout metacercariae, and within the tegument, parenchyma,

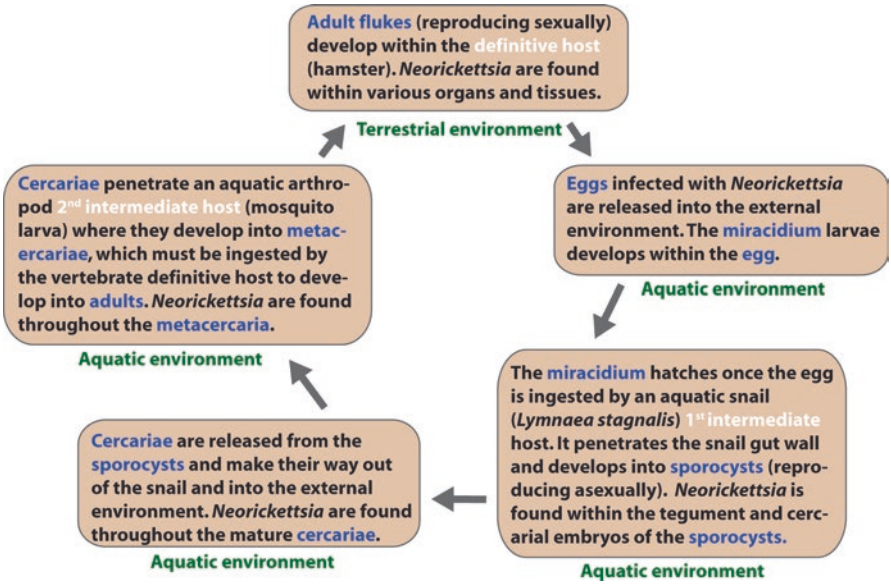

Fig. 15.7 Model life cycle of *Plagiorchis elegans* depicting the circulation pathway of *Neorickettsia* sp. The life cycle stages and hosts are identical to those in Fig. 15.5 with the natural definitive host (muskrat) replaced by the experimental host (Syrian hamster) (from Greiman et al. 2016, modified)

vitellaria, uteri, testes, cirrus sacs, and eggs of adults (Fig. 15.8). Interestingly, *Neorickettsia* were not found within the ovarian tissue, while abundant in vitelline glands and vitelline cells. Vitelline cells accumulate nutritive reserves for the developing digenean embryo. Presumably this ample supply of nutrients provides the proper environment for neorickettsiae replication. In addition, vitelline cells divide at high rate which also promotes *Neorickettsia* replication and spread to new cells. These observations suggested that vertical transmission of *Neorickettsia* within adult digeneans occurs via the incorporation of infected vitelline cells into the egg rather than direct infection of the ooplasm of the oocyte, as has been described for other bacterial endosymbionts of invertebrates (e.g., *Rickettsia, Wolbachia*).

Greiman et al. (2013) and Greiman and Tkach (2016) used PE agent as a model to study quantitative aspects of vertical transmission of neorickettsiae both in nature and the laboratory. These authors used gBlocks® synthetic DNA fragments as quantitative positive control in their quantitative real-time PCR (qPCR) assay targeting the GroEL gene. Greiman and Tkach (2016) have demonstrated that the number of bacteria significantly increased throughout all stages, from eggs to adults (Fig. 15.9) with the two largest increases in number of bacteria occurring during the periods from eggs to cercariae (33-fold increase) and from 6-day metacercariae to 48-h juvenile worms (14.5–26-fold increase). These two periods seem to be the most important for *Neorickettsia* propagation through the complex digenean life

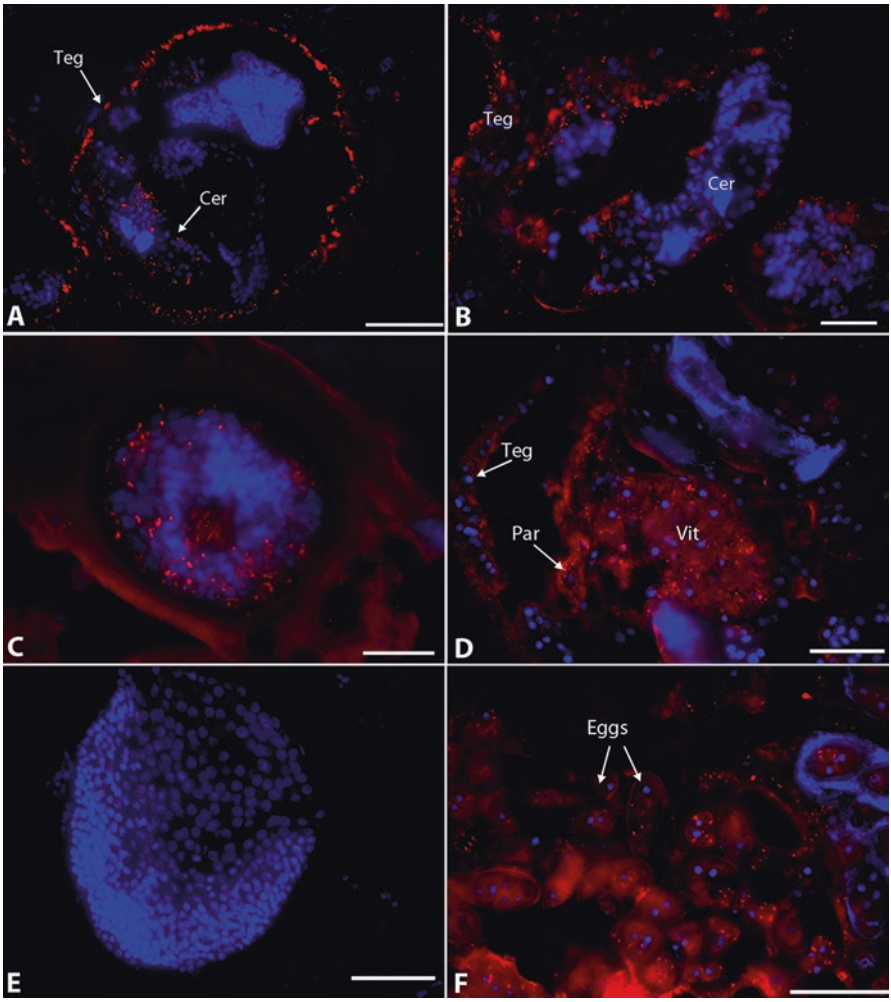

Fig. 15.8 Indirect immunofluorescence micrographs of 10 μm cryosections of sporocyst (**a**), cercariae (**b**), *Culex* mosquito larva containing mature *Plagiorchis elegans* metacercariae (**c**), mature adult fluke (**d**–**f**). *Red dots* are *Neorickettsia* sp. (PE agent); digenean nuclei are stained blue with DAPI stain; red hue on metacercarial cyst and surrounding mosquito exoskeleton are autofluorescence. Note the absence of *Neorickettsia* sp. within ovarian tissue (**e**). Abbreviations: *Cer* developing cercariae, *Teg* tegument, *Vit* vitelline glands. Scale bars: *A* = 100 μm, *B* = 25 μm, *C*–*F* = 50 μm (from Greiman et al. 2016, modified)

cycle and maturation in the definitive host. The average number of bacteria per life cycle stage of the parasite increased from 185 per individual egg to as many as 1,257,510 in an adult, a nearly 6800-fold increase (Fig. 15.9). This demonstrates a great ability of *Neorickettsia* to utilize the peculiarities of their digenean host's biology to increase their numbers and transmission potential.

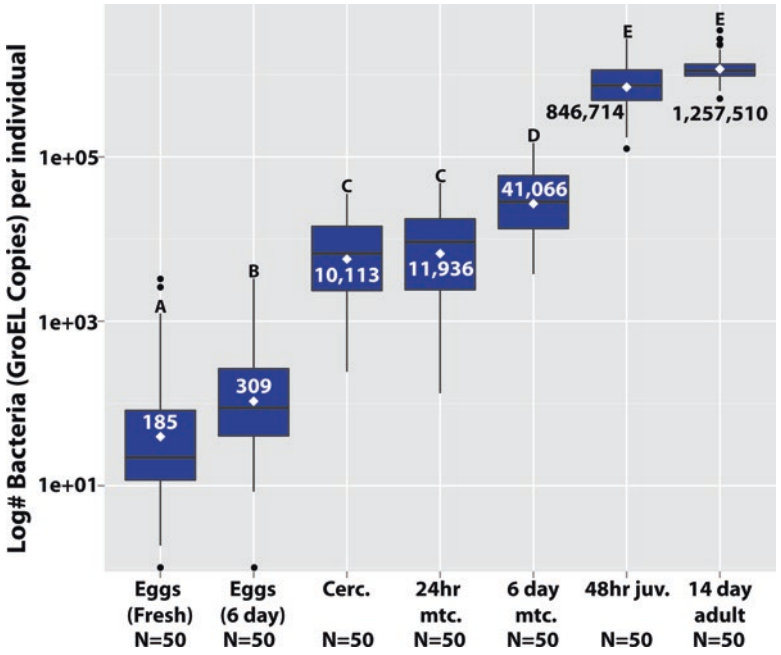

Fig. 15.9 Box plot showing the number of *Neorickettsia* sp. bacteria (GroEL copies) per individual fresh eggs, 6-day eggs, cercariae, 24-h metacercariae, 6-day metacercariae, 48-h juveniles, and 14-day adults. *Diamonds* represent mean number of bacteria, *horizontal bars* represent median values, *vertical lines* represent min–max values (from Greiman and Tkach 2016)

3.7 Neorickettsia *spp.*

Over the past several years there has been an increase in the number of studies that have identified novel genotypes of *Neorickettsia* around the world, either as a direct result of targeted screening (Seng et al. 2009; Tkach et al. 2012; Greiman et al. 2014, in press) or as an outcome of metagenomic or genomic studies (Ward et al. 2009; Mitreva et al. unpublished; GenBank LNGI01000001). As a result of these studies 18 novel genotypes of *Neorickettsia* infecting 19 species and 10 families of digeneans have been identified (Table 15.1). Unfortunately, nothing is known regarding their pathogenicity in vertebrates.

4 Associations Between Neorickettsiae and Their Digenean and Vertebrate Hosts

As shown in Fig. 15.10, neorickettsiae have now been reported from representatives of nearly all major lineages of the Digenea. The majority of *Neorickettsia* species/genotypes are known from a single digenean host species or a very narrow

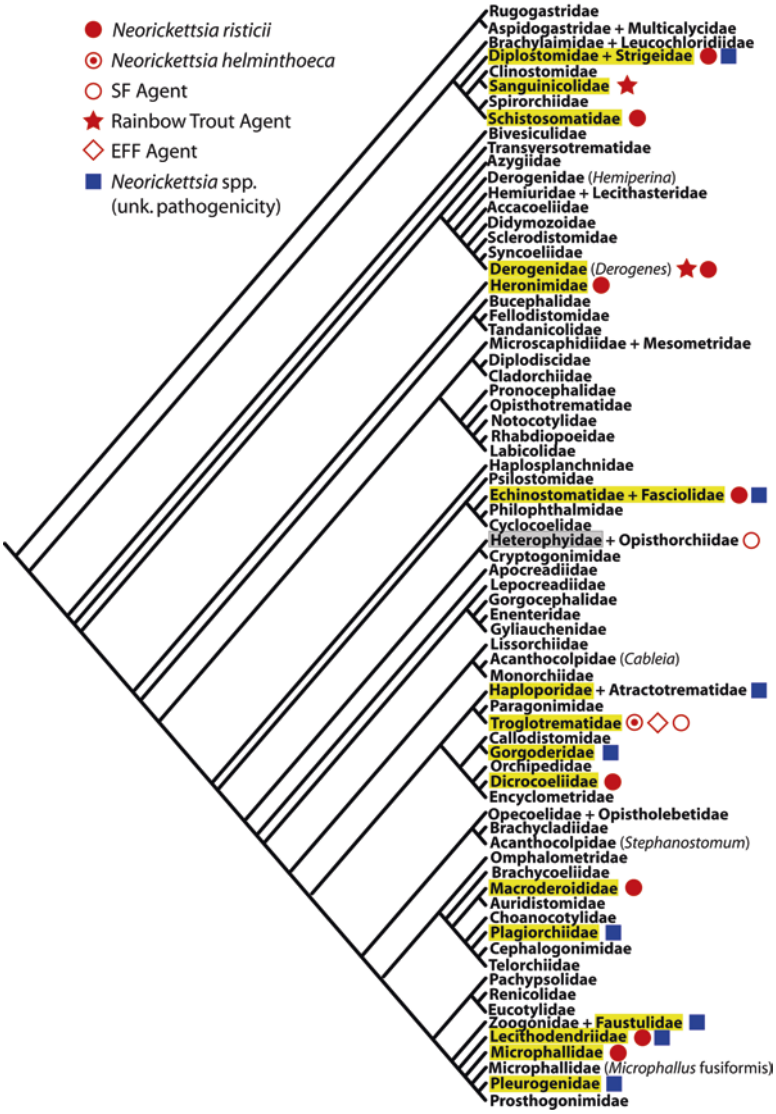

Fig. 15.10 *Neorickettsia* species/genotype records mapped on the phylogenetic tree of the digenean families (after Vaughan et al. 2012, modified)

host range, restricted to a single digenean family (e.g., SPD and EEF agents). However, a few *Neorickettsia* forms have been found in representatives of phylogenetically unrelated digenean families (e.g., *N. risticii*, rainbow trout agent, SF agent, see Fig. 15.10, Table 15.1). Exceptionally, a single digenean species, *Nanophyetus salmincola*, is currently known as the host of three different species

of *Neorickettsia*, namely, *N. helminthoeca*, *N. elokominica*, and SF agent (Greiman et al. 2016). All this indicates that neorickettsiae have had a long evolutionary history with digeneans and allows to predict that these bacteria are likely to be found in a number of additional digenean taxa. At the same time, there is no noticeable evidence of coevolutionary relationships between certain forms of *Neorickettsia* and lineages of their digenean hosts. The exact nature of the endosymbiotic relationship among neorickettsiae and their digenean hosts at physiological level is currently unclear. Most likely it is not mutualistic because there is no documented evidence of any dependency of digeneans on these endosymbionts. Usually only some individuals of the same digenean species are infected with neorickettsiae while the majority of them are not. This situation is in contrast, for example, with the mutualistic relationships among *Wolbachia* and certain species of filariid nematodes that demonstrate a clear codependency with each other (Casiraghi et al. 2005; Taylor et al. 2005; Ferri et al. 2011; Fischer et al. 2011).

Unlike the situation with the digenean host associations described earlier, some of the clades in the *Neorickettsia* phylogenetic trees (Figs. 15.11 and 15.12) show close associations among *Neorickettsia* and the definitive hosts of digeneans in which they were found. For instance, the strongly supported clade II notably comprises neorickettsiae obtained from digeneans parasitic only in fish, mostly in North America (Fig. 15.11). This clade shows the clearest association with a group of definitive hosts of digeneans. Another group that demonstrates a distinct pattern of associations with definitive hosts of digeneans is the subclade B of clade III. Digenean hosts of neorickettsiae in this subclade are parasitic in either birds or mammals with the exception of few cases where the vertebrate hosts of digeneans are unknown and *N. risticii* found in *Deropegus aspina*, a digenean that uses fish and amphibians as definitive hosts (Fig. 15.11). *Neorickettsia sennetsu*, the causative agent of the human disease Sennetsu fever in Southeast Asia, is one of the species with yet unknown digenean hosts. As suggested by Greiman et al. (2014) the 16S phylogenetic tree topology allows us to hypothesize that it should be a digenean that uses either mammals or birds as a definitive host. Furthermore, the better resolved GroESL phylogenetic tree (Figs. 15.12) illustrates that most *Neorickettsia* genotypes within the subclade uniting *N. sennetsu* and *N. risticii* utilize digeneans with arthropods (mostly insects) as second intermediate hosts. This suggests that the digenean host of *N. sennetsu* is more likely to use arthropods as a second intermediate host rather than fishes.

Based on the available records, digeneans parasitic in the complex of fish species, usually identified as *Mugil cephalus* (grey mullet), seem to harbor the highest diversity of *Neorickettsia* of all so far examined fishes. Similarly, among digeneans of terrestrial vertebrates, the most *Neorickettsia* lineages have been found in members of the family Lecithodendriidae parasitic in bats. Currently, lecithodendriids of bats have been found to harbor neorickettsiae in Africa (Egypt), North America (USA), South America (Argentina), and Asia (Thailand, Philippines).

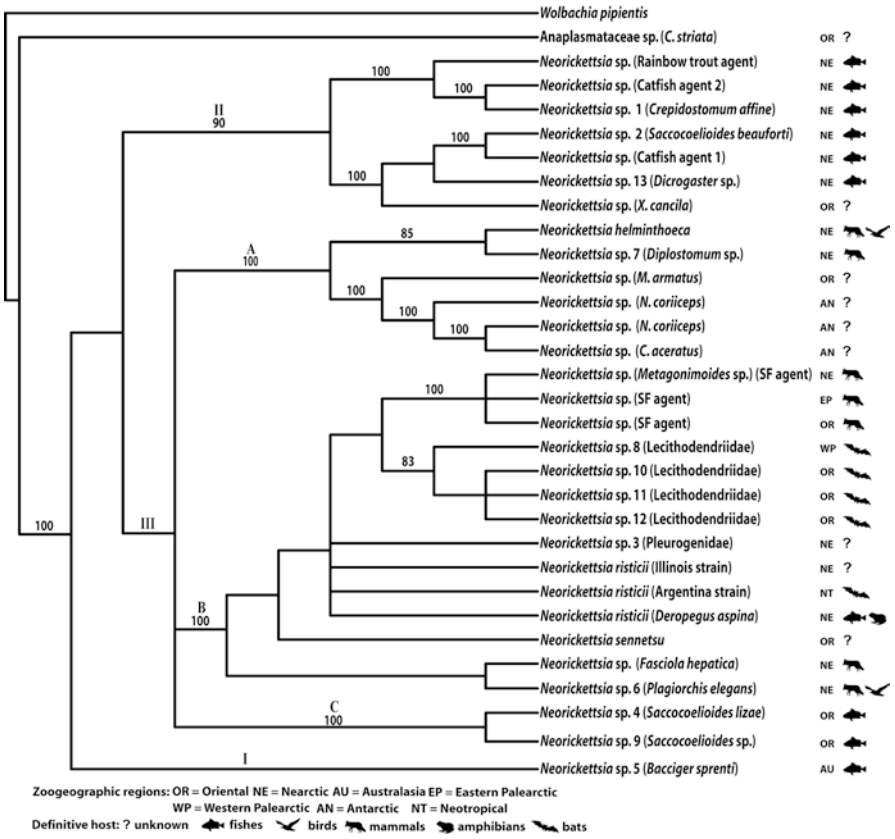

Fig. 15.11 Cladogram depicting phylogenetic interrelationships among 30 genetic lineages of *Neorickettsia* based on Bayesian analysis of partial 16S rRNA sequences. Posterior probabilities greater than 80 % are shown. Roman numerals (*I–III*) represent the different clades within the "*Neorickettsia* clade" and letters (*A–C*) correspond to the subclade within clade III. Digenean vertebrate definitive host groups are indicated by *symbols*. Zoogeographic regions are shown

5 Geographic Distribution of *Neorickettsia*

Confirmed or probable neorickettsial infections have been reported from all continents, with *N. risticii* being by far the most widely distributed and most frequently reported species of *Neorickettsia* (Dumler et al. 2005). However, many of these reports should be considered with caution unless they are confirmed by PCR/sequencing or culturing the bacteria. The majority of *N. risticii* records including all information available from Europe as well as almost all reports from Africa, Australia, and some Asian and South American countries is based exclusively on serological testing of horses with unknown or not reported vaccination or relocation histories. Mott et al. (1997) underlined that serological (usually IFA) testing is useless as a diagnostic/surveillance tool for detecting *N. risticii* in vaccinated horses

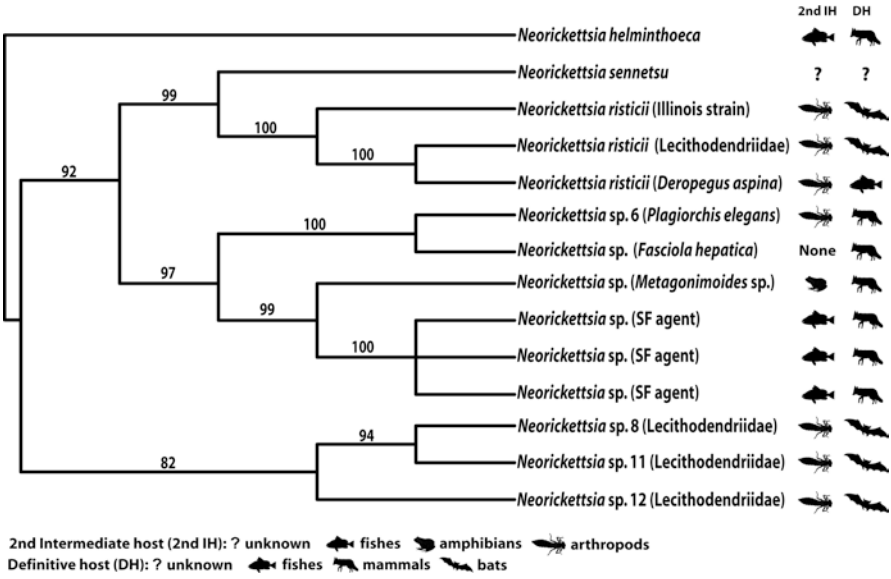

Fig. 15.12 Cladogram depicting phylogenetic interrelationships among 14 genetic lineages of *Neorickettsia* based on Bayesian analysis of partial GroESL sequences. Posterior probabilities greater than 80 % are shown. Digenean second intermediate host groups and vertebrate definitive host groups are indicated by *symbols*

and expressed the same concern as Madigan et al. (1995) regarding the reliability of reports based on serological evidence alone. Therefore, in this section and in Table 15.1 and Fig. 15.13 we consider only data resulting from application of PCR/sequencing techniques or culturing of the bacteria.

After recent discoveries of SF agent on both coasts of North America (Rikihisa et al. 2004; Greiman et al. 2014; Greiman et al. 2016; Greiman et al. in press) this species-level genetic lineage has the widest geographic range of all *Neorickettsia* (Fig. 15.13) being distributed in Japan, Southeast Asia, and both Atlantic and Pacific coasts of North America. This is also the only widely distributed form of *Neorickettsia* not linked to domestic animals. The fact that SF agent is found in digeneans parasitic in fish (including migratory fish like Pacific salmon) at one or another stage of their life cycle provides some explanation for this unusually broad distribution.

Neorickettsia risticii is the most widely spread named species of *Neorickettsia* (Fig. 15.13; Table 15.1). It is distributed throughout North America and was recently reported from Brazil, Uruguay, and Argentina. As was mentioned earlier, the records of *N. risticii* from larval stages of several digenean species in the Republic of Korea (Chae et al. 2003; Park et al. 2003) likely represent different species of *Neorickettsia* based on the relatively high level of 16S sequence divergence between Korean isolates and the typical *N. risticii* from the United States. It still means that some *Neorickettsia* species are certainly distributed in the Republic of Korea. Somewhat similar to the situation with SF agent, *N. risticii* is found in migratory vertebrate animals, although in this case flying ones such as birds and bats.

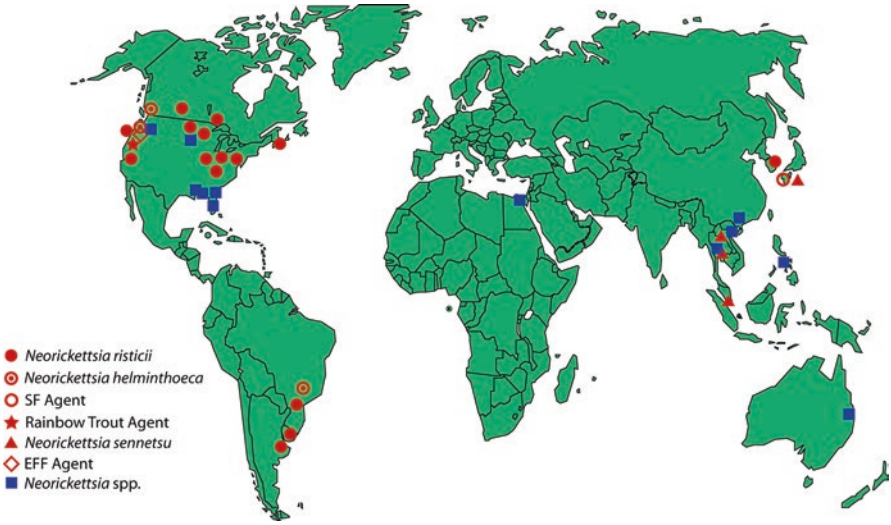

Fig. 15.13 Geographic distribution of *Neorickettsia* spp.

Another relatively broadly distributed species of *Neorickettsia* is *N. helminthoeca* that was long known from the Pacific northwest of the USA, but more recently found in other parts of the western coast of North America and Brazil. The third named *Neorickettsia* species, *N. sennetsu*, is the most widely distributed member of the genus in Asia and was found so far in Japan, Malaysia, Laos, and Thailand (Table 15.1).

The remaining *Neorickettsia* genotypes, most of them only recently identified, have very limited distribution and have been found in a single geographical area each (Table 15.1; Fig. 15.13) in North America, Australia, Africa, Japan, or Southeast Asia. The first genetically confirmed findings of *Neorickettsia* from Australia and Africa have been reported only very recently (Greiman et al. 2014; Greiman et al. in press).

Thus, using the conservative approach outlined earlier, well-documented information on *Neorickettsia* is lacking from Europe, most of South America, Africa, Australia, Asia, and nearly all island countries. Most likely, this patchy distribution pattern reflects insufficient knowledge rather than the absence of *Neorickettsia* from many geographic regions. The already documented *Neorickettsia* associations with representatives of all major phylogenetic lineages of the Digenea (Fig. 15.10, Table 15.1) suggest that these bacteria may likely be more widely distributed geographically. It is probably not accidental that the species with the widest distribution are those of recognized veterinary or medical importance. It reflects the fact that much more resources have been put into studies of these species.

The limits of geographic distribution of various *Neorickettsia* genotypes may be related to the specificity of the bacteria to their fluke hosts; most of *Neorickettsia* lineages have been found so far only in one digenean host (Table 15.1). Most digeneans demonstrate rather high specificity to their mollusk hosts, as well as, to varying

degrees, other host categories (Yamaguti 1975; Cribb et al. 2001, 2003). Thus, the distribution of most digeneans and associated neorickettsiae is limited by the range of their definitive and intermediate hosts, first of all mollusks. Considering the recent dramatic increase of the number of known *Neorickettsia* lineages and expansion of their geographic range it is safe to predict that the known geographic distribution of these endosymbionts will expand significantly in the future.

6 Molecular Detection and Identification of *Neorickettsia*

The two most commonly used approaches in *Neorickettsia* detection are the use of the indirect immunofluorescent antibodies (IFA) and polymerase chain reaction with or without subsequent sequencing. It has been shown that the IFA technique has lower specificity and sensitivity than PCR (Mott et al. 1997). Besides, IFA detects not only active infections, but also past exposure that can result from both natural infections and vaccination (in horses) and thus has a potential to be misleading. For instance, Madigan et al. (1995) provided convincing evidence of high rate of false-positive results when the IFA test was used to diagnose PHF in California. Therefore, we recommend PCR-based approach as more sensitive and specific, allowing for an exact identification of *Neorickettsia* species or genetic lineage. Nested conventional PCR reaction and real-time PCR protocols (using either SYBR Green chemistry or TaqMan probes) have been used for this purpose. Specific TaqMan probes were used for detecting two species of *Neorickettsia*, namely, *N. risticii* (Pusterla et al. 2000d) and *N. sennetsu* (Newton et al. 2009; Dittrich et al. 2015). Recently, Greiman et al. (2014) proposed a SYBR Green-based real-time protocol which allows for detection of all *Neorickettsia* species in screened samples and includes a subsequent nested PCR and sequencing of positive samples. The real-time PCR detection protocol of Greiman et al. (2014) targets the GroEL gene while the subsequent nested PCR and sequencing protocol targets the 16S gene traditionally used for differentiation among bacteria. Table 15.2 lists primers recommended for all steps. For the details of the protocols see Greiman et al. (2014).

Table 15.2 Primers recommended for the real time PCR detection of *Neorickettsia* and subsequent nested PCR and sequencing

Reaction type	Primer	Sequence (5′–3′)
Real time	groel-1500F	ATAGATCCAGCKAAGGTAGTGCGTGT
	groel-1620R	TTCCACCCATGCCACCACCAGGCATCATTG
1st round PCR	n16S-25F	TCAGAACGAACGCTAGCGGT
	n16S-1500R	AAAGGAGGTAATCCAGCCGCAGGTTCAC
Nested PCR/ sequencing	n16s-50F	TAGGCTTAACACATGCAAGTCGAACG
	n16S-1400R	CGGTTAGCTCACTAGCTTCGAGTAA
Internal sequencing primers	16S-n900F	GACTCGCACAAGCGGTGGAGTAT
	16S-n900R	ATACTCCACCGCTTGTGCGAGTC

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Part VII
Orientia

Chapter 16
Scrub Typhus and Its Causative Agent, *Orientia tsutsugamushi*

Gemma Vincent

1 Background

During World War II, Allied and Japanese Forces serving in the Asia-Pacific region were afflicted by outbreaks of a serious, acute, febrile disease. More than 16,000 cases were observed among Allied troops and 20,000 cases were recorded in members of the Japanese forces (Bavaro et al. 2005; Philip 1948). Mortality rates were variable, ranging from only 0.6 % in a large outbreak in the Schouten Islands to 35 % in a smaller outbreak in Papua New Guinea (Philip 1948). In some areas, these disease outbreaks were as disabling to a regiment as the combat itself and placed a huge burden on medical facilities (Philip 1948). For example, following a 4-day jungle exercise in Sri Lanka, 756 British soldiers were hospitalized with fever on their return to India (Philip 1948). The disease became known as scrub typhus by the Allied troops, after recognition that infection seemed to be associated with exposure to the secondary vegetation termed "scrub" (Traub and Wisseman 1974).

The disease experienced by the armed forces had been recognized as early as the third century A.D. in China (Fan et al. 1987), but it was first documented in the medical literature as tsutsugamushi disease in 1879 (Nagayo et al. 1917). The name was derived from the Japanese "tsutsuga" meaning illness and "mushi" meaning insect, in reference to the source of infection, the trombiculid mite. The tsutsugamushi disease of Japan and scrub typhus were confirmed to be same disease through studies in laboratory animals (Philip 1948). It has been known by many other descriptive and colloquial names, such as tropical typhus, mite bite fever, Japanese river fever, and Kedani fever (Hayashi 1920; Corbett 1943), but scrub typhus is the name now in common use.

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World War II accelerated research into scrub typhus, necessitated by the need to understand and prevent the disease, which was feared by the troops (Philip 1948). This research dwindled after the war, following the discovery that chloramphenicol was an effective treatment (Smadel et al. 1948). However, scrub typhus remains a serious health threat in the Asia-Pacific region. Based on its geographic distribution, an estimated one billion people are at risk of contracting the disease and more than one million cases occur each year (Watt and Parola 2003). Over 10 years ago the World Health Organization identified scrub typhus as "probably one of the most under-diagnosed and under-reported febrile illnesses requiring hospitalization in the region," and this statement is still valid today (World Health Organization Department of Communicable Disease Surveillance and Response 1999; Paris et al. 2013). The discovery of antibiotic resistant strains, the reemergence of the disease in areas where it had been absent for years and its emergence in new areas, both in the endemic region and beyond, demonstrates that research into scrub typhus is still important. This review summarizes current knowledge.

2 The Causative Agent of Scrub Typhus

2.1 Classification

The causative agent of scrub typhus is *Orientia tsutsugamushi*, an alpha proteobacterium belonging to the order *Rickettsiales* and the family *Rickettsiaceae*. When it was first isolated in 1930, it was placed in the genus *Rickettsia* and named *Rickettsia orientalis* (Nagayo et al. 1930), with a name change to *Rickettsia tsutsugamushi* shortly afterward (Ogata 1931). However, it was recognized early on that scrub typhus differed from other rickettsial diseases, in terms of the arthropod vector responsible for infection and clinical presentation (Ashburn and Craig 1908). Later research demonstrated several phenotypic differences between the scrub typhus agent and other species of *Rickettsia* (Table 16.1).

Sequencing of the 16S rRNA gene showed that while strains of *R. tsutsugamushi* shared more than 98.5 % sequence similarity, the level of similarity with other rickettsial species was only 90.2–90.6 % (Ohashi et al. 1995). This genotypic difference, along with the phenotypic differences, provided sufficient evidence for the scrub typhus agent to be reclassified in 1995 into its own genus, *Orientia* (Tamura et al. 1995). A second member of the *Orientia* genus has been proposed, although it is not yet officially recognized. The organism was isolated from an Australian patient who became infected while in Dubai in the United Arab Emirates. Sequencing of the 16S rRNA, 56-kDa and 47-kDa genes demonstrated significant genetic diversity (1.5 %, 17.7 %, and 46.9 %, respectively) from strains of *O. tsutsugamushi.* Thus, a new species name, *O. chuto* was proposed, with "chuto" derived from the Japanese for "Middle East" (Izzard et al. 2010).

Table 16.1 Phenotypic differences between the scrub typhus agent, *Orientia (Rickettsia) tsutsugamushi* and *Rickettsia* species

Feature		*Orientia* (*Rickettsia*) *tsutsugamushi* Scrub typhus agent	*Rickettsia* species	Reference
Size	Width	0.5–0.8 μm	0.2–0.6 μm	Tamura et al. (1991)
	Length	1.2–3.0 μm	0.5–2.5 μm	
Cell wall structure		Thin inner leaflet Thick outer leaflet	Thick inner leaflet Thin outer leaflet	Silverman and Wisseman (1978)
Peptidoglycan and lipopolysaccharide		Absent	Present	Amano et al. (1987)
Capsule layer		Absent	Present	Tamura et al. (1991)
Protein composition (Major protein sizes in kDa)		110, 80, 70, 60, 54–56, 46–47, 42, 35, 28 and 25	155, 120, 49, 32, 27.5, 17.5 and 16.5	Tamura et al. (1991)
In vitro susceptibility to antibiotics	Tetracyclines	Sensitive	Sensitive	Miyamura et al. (1989)
	Benzylpenicillin (1 mg/mL)	Resistant	Sensitive	
	Ofloxacin (quinolone; 1 μg/mL)	Resistant	Sensitive	

2.2 Genomics

The first full *O. tsutsugamushi* genome to be sequenced was the Boryong strain, isolated from a Korean scrub typhus patient (Cho et al. 2007). Shortly after, the genome of the Ikeda strain from a Japanese patient was fully determined (Nakayama et al. 2008) and data from a further eight Whole Genome Shotgun (WGS) projects are now available (Benson et al. 2009). The median genome size of the sequenced genomes is 2.00334 Mb as a single circular chromosome, making the *O. tsutsugamushi* genome the largest of any member of the order *Rickettsiales*, with the largest number of protein coding genes (Cho et al. 2007; Nakayama et al. 2008). Although its genome is larger than those of *Rickettsia* spp., *O. tsutsugamushi* has undergone a greater degree of reductive evolution associated with adaptation to an obligate intracellular lifestyle (Andersson and Kurland 1998). It lacks some genes involved in nucleotide metabolism, DNA recombination, DNA repair, and fatty acid biosynthesis, making *O. tsutsugamushi* more dependent on the functions of its host cell than *Rickettsia* species in which these genes are conserved (Nakayama et al. 2008).

A unique feature of the *O. tsutsugamushi* genome is the high proportion of repetitive sequences present. Repeat sequences (>200 bp) make up around 40 % of the genome, which is 200-fold greater than the density of repeats observed in the *R. prowazekii* genome and around tenfold greater than in the genomes of most other members of the order *Rickettsiales*. In fact, the repeat sequence density in the *O. tsutsugamushi* genome is more than double that of most other bacterial genomes (Cho et al. 2007; Darby et al. 2007; Nakayama et al. 2008, 2010).

The high repeat density is due to extensive amplification of mobile genetic elements that can be classified into three main types: (1) integrative and conjugative elements termed *O. tsutsugamushi* amplified genetic elements (OtAGEs), that spread by conjugative transfer between cells and integrate into the genome; (2) transposable elements (TEs); and (3) short repetitive elements (Nakayama et al. 2008). The OtAGEs make up almost 30 % of the genome (Nakayama et al. 2008, 2010) and comprise a cluster of *tra* genes for conjugative Type IV secretion systems (T4SSs), flanked on one side by an integrase gene and on the other side by putative effector genes with possible roles in signaling and interaction with the host cell (Cho et al. 2007; Nakayama et al. 2008). Conjugation systems, which facilitate the transfer of DNA between cells, are rare in intracellular bacteria. For example, the *R. bellii* genome contains a single *tra* gene operon, similar in sequence and gene organization to the OtAGEs (Ogata et al. 2006) and the plasmid of *R. felis* contains four *tra* gene fragments (Ogata et al. 2005). In contrast, the genome of the Boryong strain of *O. tsutsugamushi* has 359 *tra* genes and the Ikeda strain contains 185 remnants of OtAGEs (Cho et al. 2007; Nakayama et al. 2008). The mechanism for the amplification of the OtAGEs is not known but it has been hypothesized that the process may have been important for the adaptive evolution of *O. tsutsugamushi,* by selecting for components involved in the modulation of host cell functions (Cho et al. 2007; Nakayama et al. 2008). Since many of the genes in the OtAGEs have been pseudogenized by insertions or deletions, the genetic elements are no longer

transmissible and their current function is unclear (Cho et al. 2007). The TEs comprise five families of insertion sequence elements, four types of miniature inverted-repeat transposable elements, and a Group II intron, none of which has been identified in the genomes of *Rickettsia* spp. (Nakayama et al. 2008, 2010). They contribute 13–14 % of the genome sequence, although the amount of each type of TE varies between strains, suggesting significant decay and expansion (Nakayama et al. 2010). The huge amplification of repetitive elements has induced extensive shuffling within the *O. tsutsugamushi* genome and as such, little colinearity is observed between strains or with other rickettsial genomes (Nakayama et al. 2008, 2010). However, a set of around 500 genes is conserved between *O. tsutsugamushi* strains and five species of *Rickettsia* and thus may represent the core genes of the *Rickettsiaceae* family (Nakayama et al. 2010). In addition to intragenomic shuffling, a high rate of homologous recombination has been demonstrated to occur among *O. tsutsugamushi* strains, which also contributes to the diversification of the population (Sonthayanon et al. 2010). There is also evidence of horizontal gene transfer, through which *O. tsutsugamushi* has acquired viral and protist genes encoding ankyrin-repeat proteins and a gene from *Cyanobacteria* encoding a transposase (Georgiades et al. 2011). Overall, the genomic evolution of *O. tsutsugamushi* is unique and may be driven by the population bottlenecks created by its unique life cycle (Nakayama et al. 2008).

2.3 Strain Variation

From the early days of research into scrub typhus it was recognized that there were considerable differences in virulence between strains of *O. tsutsugamushi,* in both humans and laboratory animals (Bengston 1945). Studies using the complement fixation test (Bengston 1945; Shishido 1964), cross-neutralization (Bell et al. 1946; Bennett et al. 1947), and cross-vaccination (Rights and Smadel 1948) demonstrated serological heterogeneity between strains and three serotypes of Karp, Kato, and Gilliam were defined (Shishido 1964). Following the development of further immunological tools such as immunofluorescence assays (Bozeman and Elisberg 1967; Shirai et al. 1979) and monoclonal antibodies (Eisemann and Osterman 1985; Chang et al. 1990), and more diverse geographical studies (Elisberg et al. 1968), additional variants were identified that differed from the three original serotypes. At least nine major antigenic types are now recognized (Fig. 16.1 and Table 16.2), some of which contain subtypes (Ohashi et al. 1996).

The antigenic diversity of *O. tsutsugamushi* is due to variation of the 56-kDa outer membrane protein (Eisemann and Osterman 1985; Stover et al. 1990; Tamura et al. 1985), which is considered to be species specific (Kelly et al. 2009). Characterization of the gene encoding the 56-kDa antigen from different strains demonstrated variation in its length, resulting in the protein size ranging from 516 to 541 amino acids (Ohashi et al. 1992; Kelly et al. 2009). Comparison of different strains showed that throughout the protein sequence there are insertions, deletions,

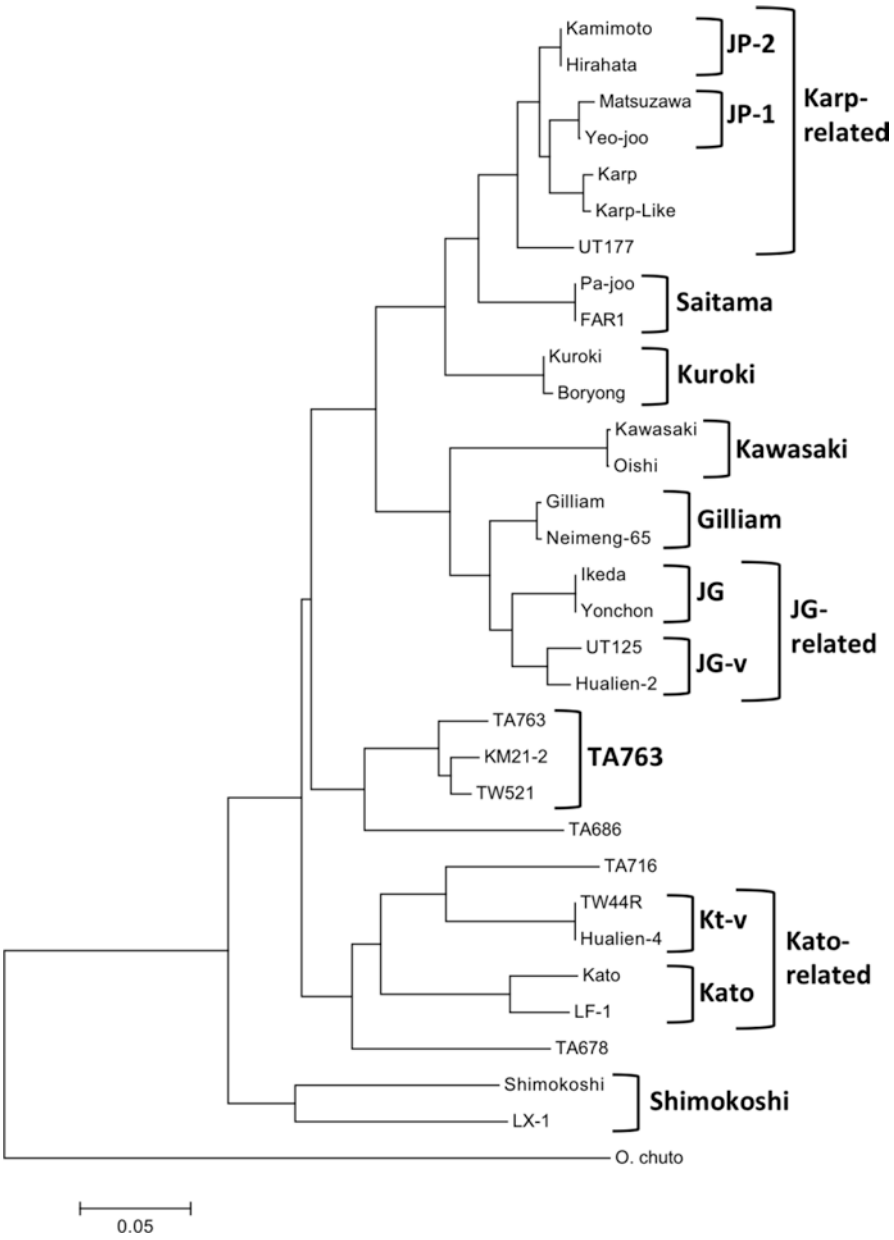

Fig. 16.1 Major antigenic types of *O. tsutsugamushi*. Phylogenetic tree of representative strains of *O. tsutsugamushi* antigenic types based on the nucleotide sequences of the 56-kDa antigen gene. The tree was constructed from a multiple sequence alignment using the neighbor-joining method, using the algorithm implemented by MEGA6 (Tamura et al. 2013). Clusters relating to the major antigenic types are indicated in *bold type*. Scale represents base substitutions per site

Table 16.2 Prevalence of major antigenic types of *O. tsutsugamushi*

Serotype		Prevalence	Comment
Karp		39.5 %	Found throughout the endemic area.
JG-related	JG-v JG	10.3 % 8.1 %	Referred to as "Gilliam-type in Japan" based on serological reactivity, although sequences are quite divergent from Gilliam. JG-v is a closely related variant.
TA763		9.6 %	First identified in Thailand but now found in China, Taiwan, India, Australia, and southeast Asia. May not occur in Japan and Korea.
Kato	Kato	6.3 %	Found throughout the endemic region.
	Kt-v	5.2 %	
Saitama		5.5 %	Similar to Karp-related strains. Found mostly in Japan but also identified in Korea and China.
Kuroki		4.1 %	Includes the fully sequenced Boryong strain.
Kawasaki		4.1 %	Found in Japan and China.
Gilliam		4.1 %	Prototype strain isolated in Myanmar but subsequently identified in isolates from India and China.
Shimokoshi		1.1 %	Highly divergent based on 56-kDa antigen gene sequence analysis.

Prevalence among collection of 271 strains for which the complete or almost complete sequence of the 56-kDa antigen gene was available (Kelly et al. 2009)

or substitutions of one or more amino acids. However, there were four particular regions in which higher rates of change were observed (variable domains (VD) I–IV) (Fig. 16.2), with protein sequence similarity between strains ranging from more than 75 % to less than 50 % (Ohashi et al. 1992). The diversity of the VDs cannot be attributed to point mutations alone and it is likely that recombination has occurred in these regions. This is supported by the fact that some strains seem to be chimeric. For example, in the Kuroki strain, VD I is similar to the Gilliam strain, VD II is Kuroki specific, VD III is similar to the Karp strain, and VD IV is similar to both Gilliam and Karp (Ohashi et al. 1992).

Since gene sequencing has become readily available, determination of the 56-kDa antigen gene sequence has become the major tool for the differentiation and classification of *O. tsutsugamushi* strains (Enatsu et al. 1999; Kelly et al. 2009). However, phylogenies based on these sequences are not accurate and different trees are obtained depending on which of the VD sequences is used (Nakayama et al. 2010). More recently, multilocus sequence typing (MLST) schemes based on seven (Sonthayanon et al. 2010; Wongprompitak et al. 2015) or 11 (Nakayama et al. 2010) housekeeping genes have been used for differentiation. A low level of congruence was observed between the MLST and 56-kDa antigen gene sequencing data (Nakayama et al. 2010; Sonthayanon et al. 2010; Wongprompitak et al. 2015), with MLST providing significantly better discrimination between isolates. For example, among a collection of 22 isolates, 15 sequence types were determined using MLST compared with only three antigenic types determined using the single locus method

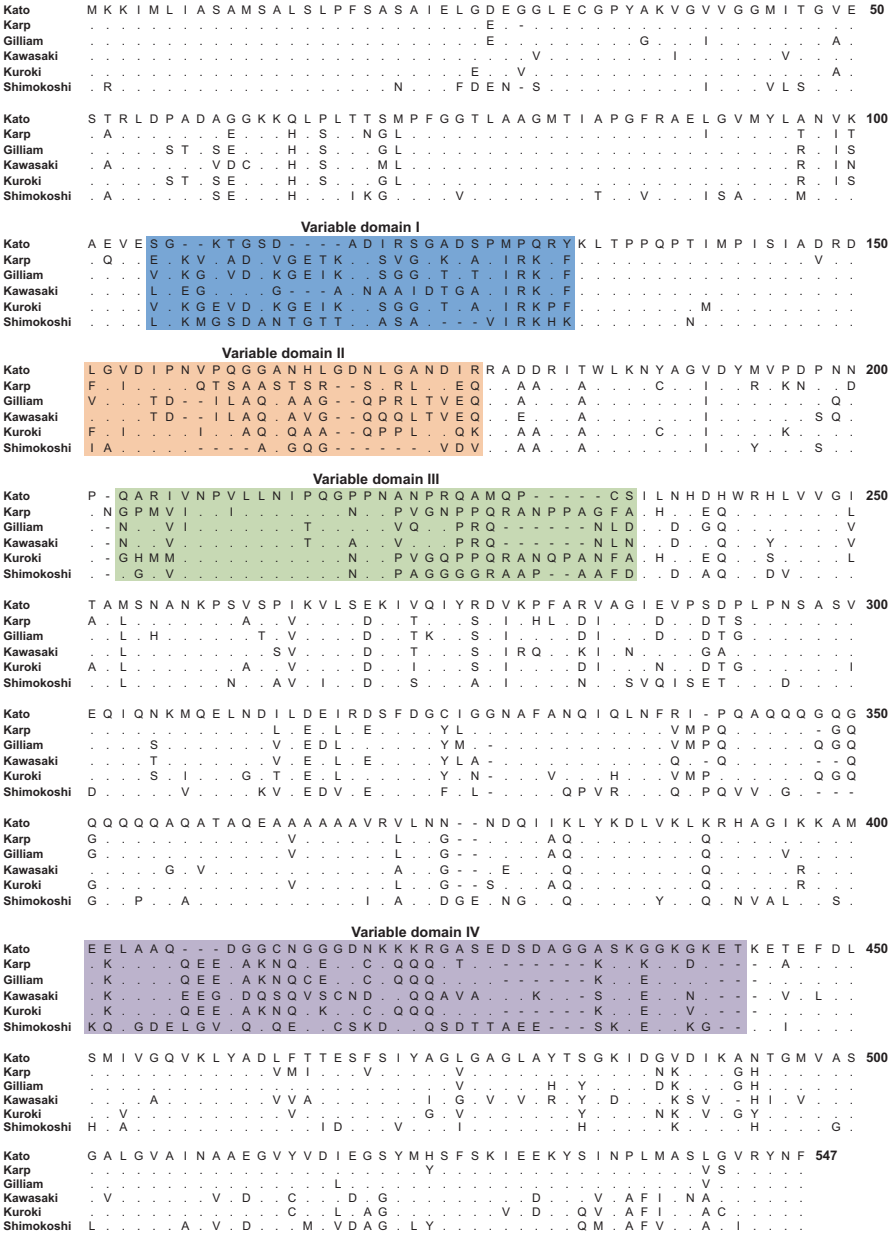

Fig. 16.2 Alignment of 56-kDa antigen sequences. Protein sequences were aligned using the ClustalW algorithm in MEGA version 6 (Tamura et al. 2013). *Colored shading* indicates the variable domains I–IV

(Sonthayanon et al. 2010). In contrast to the strongly clustered trees created from 56-kDa antigen gene sequences (Fig. 16.1), phylogenies based on MLST data have poor bootstrap support and the limited clustering observed does not correlate with the 56-kDa gene clusters (Wongprompitak et al. 2015). This limited correlation of clusters was also observed when comparing phylogenetic trees created from sequences of the *groES* and *groEL* genes with 56-kDa antigen gene phylogenies (Arai et al. 2013). While the 56-kDa antigen gene sequence may be appropriate for investigation of local outbreaks, it is less useful for the phylogenetic analysis of *O. tsutsugamushi*, since this antigen gene is under selection pressure from host immune systems (Sonthayanon et al. 2010).

Regardless of which typing method is used, there is little evidence to support the grouping of a specific antigenic or sequence type to a particular geographical location (Enatsu et al. 1999; Kelly et al. 2009; Wongprompitak et al. 2015). However, in some areas, one prevalent antigenic type is observed, which may be linked to the prevalence of a particular mite vector species (Ogawa and Ono 2008). Of the main serotypes, Karp is the most common, accounting for approximately 40 % of all isolates (Kelly et al. 2009). Many of these isolates are described as "Karp related" as they have slight differences in the 56-kDa gene sequence compared to the prototype Karp strain, but the same reading frame is maintained (Kelly et al. 2009). The prevalence of the other serotypes ranges between 1.1 and 10.3 % (Table 16.2). While some cross-reactivity is observed between antibodies raised against one serotype with antigens from another, it is important for diagnostic serological methods to incorporate a mixture of antigens to ensure cases due to an antigenically variant strain of *O. tsutsugamushi* are not missed.

2.4 Biology of O. tsutsugamushi

The typical morphological form of an *O. tsutsugamushi* cell is a short rod or coccobacillus, with a length of 1.2–3.0 μm (Tamura et al. 1991) (Fig. 16.3). It is Gram negative (Tamura et al. 1995), although the Giemsa or Gimenez stain is more useful for observing the organism grown in tissues (Gimenez 1964). Propagation of *O. tsutsugamushi* must be performed in a biosafety level 3 (BSL3) laboratory (U.S. Department of Health and Human Services 2009) and as it is an obligate intracellular bacterium, its growth in vitro requires a host cell. Successful growth has been achieved in the yolk sac membrane of embryonated chicken eggs (Clancy and Cox 1946) and a variety of cell lines including mouse lymphoblasts (Bozeman et al. 1956), mouse fibroblasts (Urakami et al. 1982), and human endothelial cells (Kim et al. 1999). However, intracellular growth studies of *O. tsutsugamushi* have been hampered by its slow replication and comparatively low yields in vitro. Studies have shown the bacterial load can be improved by the addition of up to 20 % fetal bovine serum and 0.2–0.8 μg/mL daunorubicin (an inhibitor of host cell replication) to the culture medium, although the cell doubling time is still slow, at around 9 h (Giengkam et al. 2015; Hanson 1987). While cell-free media have been developed

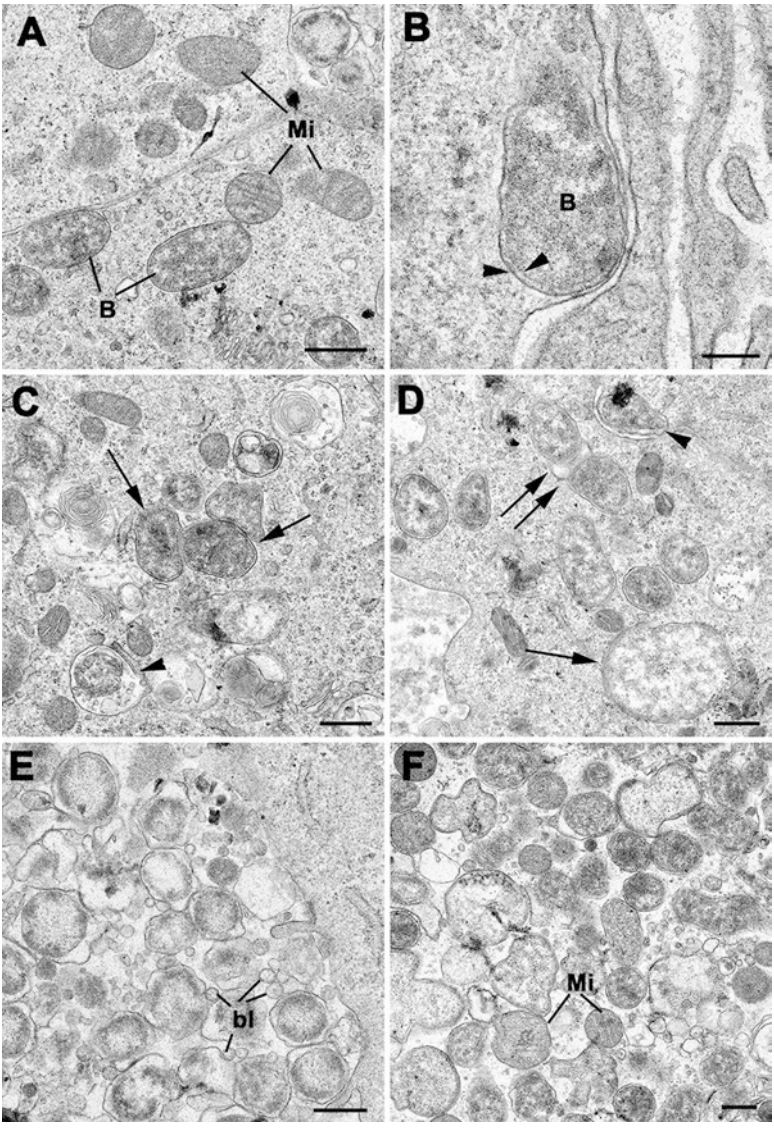

Fig. 16.3 *Orientia tsutsugamushi* morphology after infection of Vero cells (green monkey kidney epithelial cells, *O. tsutsugamushi* Thai strain UT76). (Images courtesy of Daniel Paris, Mahidol Oxford Tropical Medicine Research Unit, Mahidol University, Thailand and Centre for Tropical Medicine and Global Health, University of Oxford, UK). (**a**) Intact cytosolic *O. tsutsugamushi* (**B**), in close vicinity to mitochondria (Mi), both with and without cristae. Bar length corresponds to 500 nm. (**b**) Submembrane location of a single *O. tsutsugamushi*; the membrane protrusion is suggestive of budding in progress. Distinguishing feature of *O. tsutsugamushi*—double membrane with double leaflets (*arrowheads*). Bar length corresponds to 200 nm. (**c**) Intact *O. tsutsugamushi* (*arrows*) and degenerating *O. tsutsugamushi* in vacuoles (*arrowhead*). Bar length corresponds to 500 nm. (**d**) Vero cell with intact and free cytosolic *O. tsutsugamushi*. In the lower right is a large (approximately 2 mm in diameter) *O. tsutsugamushi* featuring a double-membrane and diffuse

for other species that were previously considered to be strictly intracellular, such as *Tropheryma whipplei* and *Coxiella burnetii*, axenic culture of *O. tsutsugamushi* has not yet been possible (Singh et al. 2013).

The in vivo target cells for human infection by *O. tsutsugamushi* are initially dendritic cells, monocytes, and macrophages in the skin at the site of the mite bite (Paris et al. 2012). The infection becomes disseminated, initially by migration of monocytes and dendritic cells to regional lymph nodes and via the lymphatic system (Paris et al. 2012), and subsequently via the blood to other organs, where endothelial cells and macrophages are the main bacterial targets (Moron et al. 2001; Keller et al. 2014).

Adherence and uptake of the bacteria by nonphagocytic host cells occurs through the process of endocytosis, which exploits host cell signaling pathways, leading to rearrangement of the host actin cytoskeleton. Several interactions between *O. tsutsugamushi* ligands and host receptor molecules have been implicated in the adhesion of bacteria to the host. The 56-kDa antigen of *O. tsutsugamushi* has been shown to bind to syndecan-4, a cell surface heparan sulfate proteoglycan (Kim et al. 2004a), and fibronectin (Cho et al. 2010; Lee et al. 2008). Inhibition of these interactions results in reduced internalization of bacteria by the host (Kim et al. 2004a; Cho et al. 2010; Lee et al. 2008). The bacterial autotransporter protein, ScaC, also binds to fibronectin, although this interaction seems only to enhance bacterial adherence, as it does not result in bacterial invasion (Ha et al. 2011). The formation of the 56-kDa antigen–fibronectin complex leads to engagement of integrin α5β1 molecules at the host cell surface and the subsequent activation of focal adhesion kinase (FAK) and Src tyrosine kinase through tyrosine phosphorylation (Cho et al. 2010). As a result, focal adhesions are formed and focal adhesion signaling adaptor proteins, such as talin and paxillin, are recruited to the site of attachment (Cho et al. 2010). In vitro observations by confocal microscopy have shown that within 10 min of infection, *O. tsutsugamushi* organisms are surrounded by membrane protrusions from the host cell surface. These are caused by rearrangements of the actin cytoskeleton, induced by the small GTPase, RhoA, which is activated following focal adhesion (Cho et al. 2010).

In addition to activation of the integrin-mediated signaling pathway, *O. tsutsugamushi* causes a transient activation of the signaling enzyme PLC-γ, which leads to the mobilization of calcium ions (Ca^{2+}) from intracellular calcium stores in the host. As a result, the host cytoplasmic calcium concentration is increased. It is cur-

Fig. 16.3 (continued) bacterial cytoplasm (*arrow*). A single *O. tsutsugamushi* is in the process of budding and potentially invading the adjacent cell (*arrowhead*). Another *O. tsutsugamushi* is in the process of mitosis and binary fission (*double arrow*). Bar length corresponds to 500 nm. (**e**) Multiple *O. tsutsugamushi* within a phagosome. There is abundant production of blebs (bl), protrusions and cleaving of the outer membrane of *O. tsutsugamushi*. Bar length corresponds to 500 nm. (**f**) *O. tsutsugamushi* can appear in various forms and sizes, with condensed or grainy and loose cytoplasm. Note the close vicinity with mitochondria (Mi). Bar length corresponds to 500 nm

rently unclear what role this plays in *O. tsutsugamushi* infection, but in vitro, inhibition of PLC-γ reduces bacterial invasion by around 80 %. It is thought that activation of the Ca^{2+} signaling pathway may occur prior to or in parallel with integrin-mediated signaling and may contribute to the rearrangement of host actin (Ko et al. 2011).

O. tsutsugamushi cells that induce host cytoskeleton rearrangement are internalized by clathrin-dependent endocytosis, similar to several other intracellular pathogens such as *Chlamydia* spp. and *Neisseria gonorrhoeae* (Chu et al. 2006). Once internalized, bacteria are contained within clathrin-coated vesicles, which move through the endocytic pathway as evidenced by colocalization of *O. tsutsugamushi* with the early endosome marker EEA1 and late endosome and lysosome marker LAMP2 (Chu et al. 2006). This colocalization is only observed early in the infection process. By 2 h postinfection, the bacteria have escaped from the endocytic compartment and are free in the cytoplasm of the cell (Chu et al. 2006). This process is pH dependent, as demonstrated by studies in which *O. tsutsugamushi* failed to traffic to late endosomes if the normal acidification of the endosome was prevented (Chu et al. 2006). The mechanism by which the bacteria promote their release from the intracellular compartment is not yet elucidated but it may involve a hemolysin or phospholipase D (Chu et al. 2006; Ge and Rikihisa 2011). The endocytic pathway is not involved during *O. tsutsugamushi* infection of professional phagocytes such as macrophages (Chu et al. 2006). Instead, the bacteria become internalized within a phagosome, from which some may escape into the host cytoplasm (Rikihisa and Ito 1979).

O. tsutsugamushi free within the cytoplasm move from the periphery of the cell to the microtubule organizing center in the region around the nucleus, using the microtubule network. Bacterial movement is mediated by the motor protein dynein. This is in contrast to other rickettsiae, which move within the cytoplasm using the host cell actin (Kim et al. 2001). Although intranuclear replication of *O. tsutsugamushi* has been observed (Urakami et al. 1982; Pongponratn et al. 1998), replication occurs more typically in the perinuclear region of the cytoplasm by binary fission (Urakami et al. 1984) (Fig. 16.3d). As the host cell becomes more heavily infected with *O. tsutsugamushi,* micro-colonies are observed, which may be biofilm-like structures, aggregated by matrices of the bacterial polysaccharide antigen, NT19 (Lee et al. 2009). In vitro, by 72 h postinfection, *O. tsutsugamushi* cells are observed throughout the host cell cytoplasm, including at the cell periphery. The bacteria push on the host cell membrane from the inside (Fig. 16.3b, d) and as the infection proceeds, numerous bud-like projections containing bacteria are observed on the host cell surface (Urakami et al. 1984). The bacteria are released from the host cell, either in host membrane coated buds (Ewing et al. 1978) or as free bacteria, following lysis of the host (Rikihisa and Ito 1979). Both forms have been shown to be capable of infecting other new cells, although one form may be more infective than the other for some host cell types. For example, in a study performed in mice, mesothelial cells of the peritoneal cavity were able to phagocytose the host membrane coated vesicles containing *O. tsutsugamushi* but not uncoated bacteria (Ewing et al. 1978). A similar budding process has been observed in mites infected

with *O. tsutsugamushi*, where bacteria are released from the cells of the mite salivary glands, when triggered by mite feeding (Kadosaka and Kimura 2003).

The interaction of *O. tsutsugamushi* with its host is a complex process. A study of human monocytes showed that the expression of more than 4500 genes was altered in response to infection by *O. tsutsugamushi.* The most up-regulated genes (>20 % enrichment) were those involved in the immune response and more than 15 % of these genes were cytokines or chemokines (Tantibhedhyangkul et al. 2011). While a normal host response elicits the production of cytokines, if this production is excessive it can cause a severe systemic disorder in the host (Ge and Rikihisa 2011). The hyperproduction of cytokines and chemokines has been observed in a scrub typhus susceptible mouse model (Yun et al. 2005) and in scrub typhus patients, and is likely linked to some of the systemic symptoms observed (Kramme et al. 2009; Chung et al. 2008). Infection with *O. tsutsugamushi* can result in the subversion of functions within the host cell, which promotes bacterial survival. For example, the process of autophagy plays an important role in both the innate and adaptive immune response against several intracellular pathogens (Choi et al. 2013). Although *O. tsutsugamushi* induces autophagy, it actively escapes the autophagosomes, therefore its growth is unaffected by the process (Choi et al. 2013; Ko et al. 2013). Whole genome sequencing of *O. tsutsugamushi* revealed a large number of genes encoding proteins with eukaryotic ankyrin-repeat (Ank) domains (Cho et al. 2007; Nakayama et al. 2008). These proteins have been found in other intracellular bacteria such as *Coxiella burnetii* and *Anaplasma phagocytophilum*, in which they have been implicated in the regulation of host cell processes. Recent studies have shown that the *O. tsutsugamushi* Ank proteins are substrates for the Type I secretion system (Beyer et al. 2015; Min et al. 2014; Viebrock et al. 2014). Several of these proteins traffic to the endoplasmic reticulum of the host cell, where they may be involved in modulation of the host response to infection (Viebrock et al. 2014) and one (Ank9) interacts with the host cell polyubiquitination machinery, although the host cell protein targets are yet to be determined (Beyer et al. 2015). *O. tsutsugamushi* may also affect the metabolism of its host. For example, in L929 cells, infection with *O. tsutsugamushi* resulted in altered lipid metabolism, with the accumulation of organelles capable of storing triglycerides, known as lipid droplets (Ogawa et al. 2014). The role of this alteration has not yet been determined, but lipid droplets are essential for the growth of *Chlamydia trachomatis* (Cocchiaro et al. 2008), and may also be involved in the growth or survival of *O. tsutsugamushi* (Ogawa et al. 2014).

3 Disease Transmission

Like other rickettsial diseases, scrub typhus is arthropod borne. Its vector is the larval stage of more than 50 species of trombiculid mites belonging to several genera, but species of the genus *Leptotrombidium* are considered to be the primary cause of disease transmission in most countries (Lee et al. 2011). These larvae (Fig. 16.4) are commonly referred to as chiggers.

Fig. 16.4 Larval (chigger) stage of the trombiculid mite. Micrograph of an unfed larva of the species *Eutrombicula hirsti*. Although not a vector of scrub typhus, this larva is typical of the trombiculid mites. (Image courtesy of Stephen Frances, Vector Surveillance and Control, Australian Army Malaria Institute)

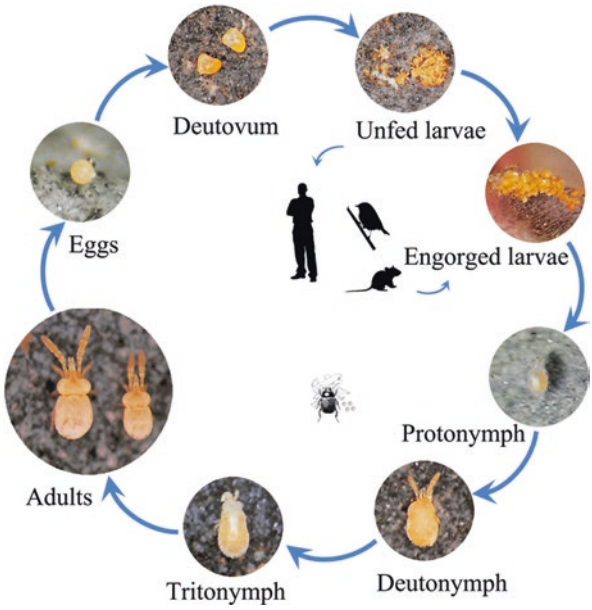

Fig. 16.5 Life cycle of trombiculid mites. The life cycle comprises four main stages (egg, larva, nymph, and adult) with three intermediate, quiescent stages. Humans are an incidental host of the larvae, which more commonly feed on mammals or small birds (Santibáñez et al. 2015)

The life cycle of trombiculid mites encompasses four main stages: egg, larva, nymph, and adult (Fig. 16.5). Females lay their eggs loosely in soil over a period of 6–12 weeks, at a rate of one to five eggs per day. After a break of a similar time, a second phase of oviposition takes place. During this whole 3–5 month period, a single female will lay around 400 eggs. The egg stage lasts only 5–7 days, then the shell ruptures, exposing the deutovum (an immature, quiescent form), which develops into the larva within another week. The six-legged larvae that emerge are barely visibly to the naked eye. Within 2 days of emergence, they are ready to feed, with most species attaching to any mammal or bird with which they can make contact.

The larvae feed on serum exudate and cellular debris rather than blood, taking 2–12 days to become fully engorged. After detaching from their host, they enter a second quiescent, pupa-like phase (protonymph), and develop into the eight-legged nymph within 7–10 days. The nymphs do not require a host, but instead feed on the eggs of other arthropods and quiescent or deceased soft-bodied insects. Within about 2 weeks, the nymphs enter another quiescent phase (tritonymph) and emerge as adult mites around 2 weeks later. Male adults deposit spermatophores, which are collected by the adult females, and oviposition begins within a fortnight. The life cycle of most *Leptotrombidium* mites typically takes 2–3 months, but it may be as long as 8 months in some species. Adult mites live for around 4–6 months, although some have been observed to survive for more than 14 months in the laboratory (Neal and Barnett 1961; Traub and Wisseman 1974; Walker et al. 1975).

Since only the larvae bite humans, for the mite to act as a vector for scrub typhus it is essential that the causative bacteria are transmitted trans-stadially to the subsequent nymph and adult stages, then transovarially from the female to the eggs (Burgdorfer and Varma 1967). This results in a new population of infected larvae capable of transmitting disease. The transovarial transmission of *O. tsutsugamushi* has been demonstrated and studied in many species of trombiculid mites (Rapmund et al. 1969, 1972; Traub and Wisseman 1974; Roberts and Robinson 1977; Frances et al. 2001; Phasomkusolsil et al. 2009; Shin et al. 2014). This process is very efficient, with transovarial transmission rates (percentage of infected females that transmit the bacteria to their progeny) often as high as 100 %. Filial infection rates (percentage of infected larvae derived from a single infected female) are more variable and seem to vary between parents (Rapmund et al. 1969; Urakami et al. 1994a). In laboratory reared mites, three main patterns of filial infection rates have been observed: (1) all the progeny are infected, (2) all except one or two individuals are infected, and (3) the offspring are mostly uninfected (Rapmund et al. 1969). In some laboratory-reared colonies, filial infection became less efficient over time. Commonly, 100 % of the first generation of larvae from *O. tsutsugamushi* infected females was also infected, whereas in later generations, the overall filial infection rate dropped to less than 65 % (Urakami et al. 1994a; Frances et al. 2001; Phasomkusolsil et al. 2009).

In infected mites, *O. tsutsugamushi* is distributed throughout the body and found in most tissues, although not all studies have demonstrated its presence in the muscles (Urakami et al. 1994a, b; Wright et al. 1984). Particularly high concentrations of the organism are found in the salivary glands of the larvae and in the salivary glands, excretory bladder, epidermal layer, digestive tissues, and reproductive organs of adults (Wright et al. 1984; Urakami et al. 1994a). Although the bacteria have been observed in the testes of infected adult males, they are not transmitted to the spermatophores (Takahashi et al. 1988; Urakami et al. 1994b), thus only infected females are capable of passing the infection to their offspring. In some species, infection with *O. tsutsugamushi* has been shown to affect the development of infected mites. Infected larvae of *L. chiangraiensis* and *L. imphalum* were shown to feed and detach from their host quicker than larvae that were not infected. This may be advantageous as the period where the mites are vulnerable to antimite behavior

by the host is reduced (Phasomkusolsil et al. 2012). Egg production in several species of mites is higher when the adult females are infected (Roberts et al. 1977; Frances et al. 2001), again conferring an advantage to the mite, by increasing the number of progeny. However, in other species, a reduction in both egg production and hatching is observed in infected mites (Roberts et al. 1977; Phasomkusolsil et al. 2012; Shin et al. 2014), which would be expected to decrease their survival. In some species of mite studied, infection with *O. tsutsugamushi* alters the sex ratio of the offspring from around 2:1 in favor of females, to exclusively females (Roberts et al. 1977; Shin et al. 2014). This phenomenon has not been observed in uninfected mites.

The primary hosts of the trombiculid mite larvae are wild rodents. Many studies have demonstrated the presence of *O. tsutsugamushi* in these animals (Jackson et al. 1957; Walker et al. 1973; Glazebrook et al. 1978; Ishikura et al. 1985; Lerdthusnee et al. 2008; Cosson et al. 2015) and the larval mites successfully transmit the bacterium to their rodent hosts (Frances et al. 2001; Lerdthusnee et al. 2002). Although the larval acquisition of *O. tsutsugamushi* from an infected animal and subsequent trans-stadial transmission have been demonstrated, the infection is very rarely passed on to the mites' offspring (Traub et al. 1975; Walker et al. 1975; Takahashi et al. 1994). It is thought that the bacteria may not penetrate the walls of the gut, thus never reaching the correct part of the mite's body to be transmitted by the transovarial route (Walker et al. 1975; Traub et al. 1975). The reservoir of a disease must be capable of maintaining "a regular or permanent source of infection in nature" (Traub et al. 1975). Since uninfected mites acquiring *O. tsutsugamushi* from their hosts are not able to transmit the bacteria to another host, wild rodents are not a reservoir of scrub typhus. Instead, trombiculid mites are considered to be both the disease reservoir and vector. However, rodents are important in the ecology of scrub typhus and it is likely that infected rodents provide the explanation for the occurrence of multiple strains of *O. tsutsugamushi* within individual larvae (Shirai et al. 1982; Frances et al. 2000).

4 Epidemiology

Traditionally, the worldwide distribution of scrub typhus has been defined by the 'tsutsugamushi triangle.' This describes the region in which the disease is endemic and encompasses an area of approximately 13 million square kilometers, with the northern most point in Korea and the far east of Russia, reaching to tropical northern Australia in the south and Afghanistan in the west (Paris et al. 2013) (Fig. 16.6). However, in recent years, this concept has been challenged by cases of the disease outside of the endemic region. The aforementioned case of scrub typhus caused by the proposed new species *O. chuto* was acquired in Dubai in the United Arab Emirates, around 500 km west of the previously recognized area (Izzard et al. 2010) and in 2006, a patient bitten by a leech on a Chilean island developed a scrub typhus-like illness, with an *Orientia*-like agent confirmed by DNA

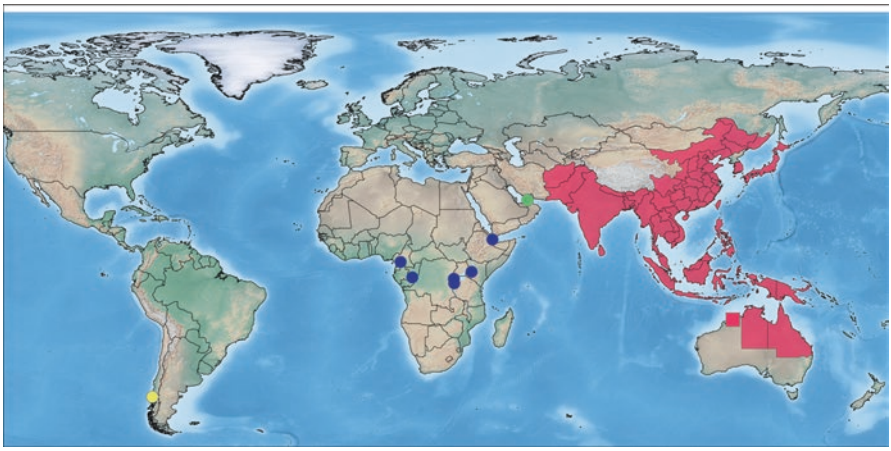

Fig. 16.6 Worldwide distribution of scrub typhus. Map indicates the regions in which scrub typhus is known to be endemic (*red*) and the locations of scrub typhus cases caused by *O. chuto* (*green dot*) and an Orientia-like species (*yellow dot*). Possible locations of scrub typhus infection in Africa are also indicated (*blue dots*)

sequencing (Balcells et al. 2011). If the leech is shown to be an alternative vector for scrub typhus, the distribution of the disease may be increased. A further 3 confirmed cases of scrub typhus have more recently been reported from the same island, in patients that had never travelled outside of Chile, therefore the infection may now be endemic in this region (Weitzel et al. 2016). There have also been reports of scrub typhus cases and seroprevalence studies in which infection with *O. tsutsugamushi* was believed to have occurred in Africa (Osuga et al. 1991; Ghorbani et al. 1997; Thiga et al. 2015; Luce-Fedrow et al. 2015). It is important for clinicians in nonendemic countries to consider the disease in travelers with compatible symptoms returning from the endemic region, and in light of these recent cases to be aware that scrub typhus may also be acquired outside of the 'tsutsugamushi triangle.'

Humans become infected with *O. tsutsugamushi* via the bite of an infected chigger, when they encroach on the habitats in which the mites are found. Therefore, the epidemiology of scrub typhus is closely linked to the distribution and behavior of the trombiculid mite species that are vectors of the disease. Unsurprisingly, positive correlations have been demonstrated between the number of human scrub typhus cases in an area with both the coincidence of mite vector species (Ishikura et al. 1985; Roh et al. 2014) and the prevalence of *O. tsutsugamushi* infection in wild rodents (Ishikura et al. 1985; Lin et al. 2014). The description of the disease as scrub typhus is a misnomer as trombiculid mites are not restricted to the scrub vegetation of subtropical regions but are found in a diverse range of climates and habitats. These include subarctic regions, semidesert (Traub and Wisseman 1968), woody vegetation (Traub and Wisseman 1974), deep jungle (Cadigan et al. 1972), and rice paddies (Tanskul et al. 1998). Some of the scrub typhus vector mite species have a

very restricted distribution. For example, *L. arenicola* is only found in Malaysia (Upham et al. 1971). Other species, such as *L. deliense*, appear to be more adaptable and their distribution is more widespread (Traub and Wisseman 1974). Within the areas in which the mites are found, their distribution can be highly focal. Localized units of mites are termed "mite islands" and they range in size from a few centimeters to more than a meter (Traub and Wisseman 1968). They can be explained by the observation that the larvae are rarely observed individually, but occur mostly as clusters, which wait on stems or leaves to attach to a host (Gentry et al. 1963).

The seasonal occurrence of scrub typhus varies with the climate in different countries. For example, on the Korean peninsula, the peak incidence is during October and November (Noh et al. 2013), whereas in southern China, the number of cases peaks first in the summer months of June and July, then second in September and October (Wei et al. 2014a). In contrast, in some areas of Taiwan, no seasonal variation in the number of cases is observed (Tsai and Yeh 2013). Early observations of *L. deliense* and *L. akamushi* in Malaysia showed that the larvae in their natural habitats were sensitive to changes in temperature and humidity (Gentry et al. 1963), thus seasonal variation in disease occurrence is linked to fluctuations in temperature and rainfall. Many studies have described increased numbers of chiggers during periods of increased rainfall (Traub and Wisseman 1974), therefore scrub typhus outbreaks are often associated with monsoon and rainy seasons (Gurung et al. 2013) or periods of heavy rain (Faa et al. 2003). In temperate regions, scrub typhus cases are correlated more with temperature variations than with rainfall (Van Peenen et al. 1976; Kuo et al. 2011). There is evidence that climate change may be affecting the temporal occurrence and number of scrub typhus cases in some areas. For example, in Pingtan Island in eastern China, the known vector of scrub typhus is *L. deliense*, which previously appeared in late May, resulting in cases of scrub typhus in the summer. Since 2000, several cases of the disease have been reported in the spring, with mites observed as early as March. This could be linked to a rise in the average March temperatures since 1997 (Cao et al. 2006). Increases in temperature, sunshine, and rainfall were also linked to an increase in scrub typhus incidence in some areas of northern and southern China (Li et al. 2014; Yang et al. 2014).

Traditionally, scrub typhus is considered to be a disease associated with agricultural activities in rural areas (Kuo et al. 2011). Seroprevalence and outbreak studies have identified various occupational and behavioral risk factors for exposure to *O. tsutsugamushi*. These include being a farmer (particularly on dry, cultivated land), working in vegetable fields, bundling waste straw, living at the edge of a village, sitting on grass while taking breaks, and having close contact with rats (Mathai et al. 2003; Kweon et al. 2009a; Vallée et al. 2010; Kuo et al. 2011; Lyu et al. 2013; Wei et al. 2014b; Hu et al. 2015). A higher prevalence of antibodies against *O. tsutsugamushi* has been observed in older people (>50 or 60 years) in several studies (Bang et al. 2008; Brown et al. 1978a; Kuo et al. 2011; Vallée et al. 2010; Zheng et al. 2015), probably reflecting increased opportunities for exposure over the course of their lifetime. Many studies have demonstrated an increased risk of exposure for females compared to males (Bang et al. 2008; Kweon et al. 2009b; Kuo et al. 2011; Noh et al. 2013; Zheng et al. 2015), which may be linked to differences in the work-

ing behaviors of men and women in some areas. In South Korea, for example, men tend to work in the rice fields, where they use tools in a standing position. Women are more likely to be employed in dry fields, where they work with bare hands, typically in a squatting position in which they are more likely to come into contact with infected mites (Kweon et al. 2009b). However, in some areas such as Japan, there is no significant difference in exposure risk between the two sexes and equal numbers of scrub typhus cases are seen in males and females. This is believed to reflect cultural differences between different countries in terms of work and clothes (Bang et al. 2008). Military personnel deployed in endemic areas also have a high risk of contracting scrub typhus, due to their exposure to vegetation harboring infected mites. Subsequent to the aforementioned cases observed during World War II, outbreaks occurred during the Vietnam conflict and numerous cases have been recorded during training exercises and humanitarian operations (Corwin et al. 1999; Bavaro et al. 2005; Likeman 2006).

There is evidence to suggest that the epidemiology of scrub typhus is changing. Certainly, the disease is becoming urbanized and can no longer be considered a problem limited to rural areas. In recent years, numerous cases and outbreaks have been recognized in urban patients (Strickman et al. 1994; Wei et al. 2014a, b; Sethi et al. 2014; Park et al. 2015). One such outbreak, associated with a city park in China resulted in four deaths (Wei et al. 2014b). The urbanization of scrub typhus can be attributed to three main factors. First, the incursion of expanding human populations into agricultural or previously uninhabited areas has resulted in deforestation and the clearing of land, creating more suitable habitats for the vector mite species (Strickman et al. 1994; Vallée et al. 2010). Second, infected chiggers have been found in central urban locations, possibly due to their spread from surrounding areas (Park et al. 2015). Third, changes in the behavior of urban residents have made their contact with trombiculid mites more likely. For example, the working week in Korea was reduced to 5 days in 2004, allowing urban workers more time for recreational activities such as golf and climbing, or for agricultural activities such as harvesting chestnuts (Kweon et al. 2009b). In rural areas, changes in land use may affect scrub typhus epidemiology. For example, following Taiwan's admission to the World Trade Organization, its rice market was exposed to foreign competition, leading to the abandonment of many rice paddies and reduced plowing in agricultural regions. This resulted in overgrowth of secondary vegetation and an increase in rodents and chiggers in these areas, increasing the risk of scrub typhus transmission (Kuo et al. 2012).

The incidence of scrub typhus is increasing in many countries. In part this may be due to improvements in diagnostic testing and better awareness. However, it is clear that the disease has reemerged in areas where cases had not occurred for many years (Lewis et al. 2003; Khan et al. 2012; Sethi et al. 2014) and that it is newly emerging in regions where it had not been recognized previously (Zhang et al. 2010; Hu et al. 2015). For example, in Japan where much of the early scrub typhus research was carried out, the disease was apparently absent for a period of around 10 years prior to its reemergence in 1976, with a subsequent increase in case numbers and expansion of endemic areas (Ishikura et al. 1985). Particularly rapid increases in

disease incidence have been seen in some countries in recent years (Kweon et al. 2009b; Li et al. 2013; Zhang et al. 2013). In South Korea there were almost four times more cases in 2013 compared with 2001 (Lee et al. 2015a) and in China, 12.8 times more cases were reported in 2014 compared with 2006 (Wu et al. 2016). It is important to understand the changing epidemiology of scrub typhus to inform public health policies in both traditional and newly recognized endemic areas.

5 Scrub Typhus, the Disease

5.1 Clinical Manifestations

Scrub typhus presents as a mild disease in some patients, whereas others suffer from a more severe illness, which may even result in death. The bite of the chigger responsible for the transmission of *O. tsutsugamushi* to humans is usually painless and goes unnoticed by the patient (Watt and Parola 2003). Person-to-person transmission of scrub typhus is rare but a few instances have been recorded. Routes of transmission include needle-stick injuries via the placenta from mother to baby and stem cell transfusion (Wang et al. 1992; Kang et al. 2010).

In the days following infection, a small, (2–3 mm), reddish lesion may develop at the bite site and swelling may occur in the adjacent lymph nodes (Hayashi 1920). However, normally the first sign of illness is the sudden onset of a fever accompanied by nonspecific symptoms such as chills, headache, coughing, myalgia, nausea, diarrhea, and vomiting (Corbett 1943; Lee et al. 2013). The incubation period of scrub typhus is typically 7–10 days but can vary between 6 and 21 days.

In some patients, the small lesion at the bite site becomes larger, undergoing necrosis at the center and developing a blackened crust. This larger lesion is known as an eschar and it resembles a cigarette burn (Watt and Parola 2003) (Fig. 16.7).

Eschars are usually found on parts of the body that tend to be warm and damp, such as the groin and axilla and often where pressure from clothing occurs, such as the waistband (Irons and Armstrong 1947). Differences in the distribution of eschars on males and females have been observed. In a study of 162 patients with eschars, the primary area for eschar development in males was within 30 cm below the umbilicus, whereas in females, the lesions were most prevalent on the front chest, above the umbilicus (Kim et al. 2007a). Another study demonstrated that preferential eschar development sites varied in different geographic areas (Zhang et al. 2012). Multiple eschars on a single scrub typhus patient are rare but have been documented (Kim et al. 2007a; Kaushik et al. 2014). Eschars are not present in all cases of scrub typhus and incidence rates are extremely variable. A low prevalence of 1–4 % has been recorded in several case series from India (Mathai et al. 2003; Sharma et al. 2005; Sethi et al. 2014), although another Indian study observed eschars in 45 % of the patients (Chrispal et al. 2010). In cohorts from other Asian countries, eschars have been present in a higher proportion (>60 %) of the patients (Sirisanthana et al. 2003; Kim et al. 2010; Hu et al. 2015). In addition to the eschar, some scrub typhus

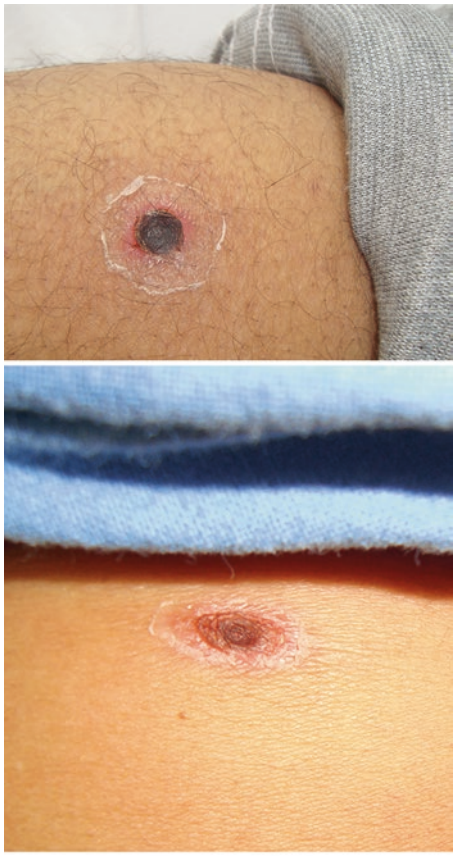

Fig. 16.7 Examples of eschars on scrub typhus patients. Images courtesy of Munegowda Koralur; Kasturba Medical College, Manipal University, India

patients develop a maculopapular rash on the trunk, which appears 5–8 days after the onset of fever. The rash may extend to the arms and legs later in the infection (Irons and Armstrong 1947; Jeong et al. 2007). Other signs of the disease include generalized lymphadenopathy, acute hearing loss, conjunctival congestion, hepatomegaly, and splenomegaly (Noad and Haymaker 1953; Premaratna et al. 2006; Chrispal et al. 2010; Zhang et al. 2012).

In some patients, the clinical course of scrub typhus is more severe and a range of serious complications has been reported involving organs of the pulmonary, cardiac, abdominopelvic, and central nervous systems. These include interstitial pneumonia (Choi et al. 2000), acute respiratory distress syndrome (ARDS) (Park et al. 2000; Wang et al. 2007), myocarditis (Levine 1946), acute cholecystitis (Lee et al. 2015b), renal failure (Yen et al. 2003; Kim et al. 2010), acute kidney injury (Attur et al. 2013), gastrointestinal bleeding (Irons and Armstrong 1947), meningitis or meningoencephalitis (Kim et al. 2013), severe disseminated intravascular coagulation (Ono et al. 2012), and septic shock (Sethi et al. 2014). The diversity of these clinical manifestations is explained by the basic pathology of *O. tsutsugamushi*

infection. The bacteria cause a focal or disseminated vasculitis due to the destruction of the endothelial cells (Choi et al. 2000; Chrispal et al. 2010). In cases of severe scrub typhus, it is not uncommon for multiple organs to be affected. For example, in a cohort of 116 patients with severe scrub typhus, 85 % experienced a dysfunction of three or more organ systems (Griffith et al. 2014). Risk factors that correlate with the development of severe disease include older age (>60 years), the absence of an eschar, low serum albumin (≤3.0 g/dL), elevated leukocytes (>10,000 μL), the presence of interstitial pneumonia, and elevated plasma inflammatory markers such as YKL-40 (Song et al. 2004; Chrispal et al. 2010; Kim et al. 2010; Otterdal et al. 2014). There is also evidence of a relationship between bacterial load and severity of the disease. A study of 155 Thai scrub typhus patients demonstrated a positive correlation between the concentration of *O. tsutsugamushi* in the blood taken on admission to hospital and the duration of illness, presence of an eschar, and hepatic enzyme levels. The bacterial load in patients that died was significantly higher than in those that recovered from their illness (Sonthayanon et al. 2009). A large systematic review of more than 19,000 untreated scrub typhus patients in 89 case series showed that overall, median mortality from the disease was only 6 %, although a wide range from 0 to 70 % was observed (Taylor et al. 2015). Variability may be due to numerous factors including the infecting strain, geographic area, and host factors (Taylor et al. 2015). Mortality rates in patients with severe complications are often higher. For example, studies of patients with ARDS have demonstrated mortality rates between 22 and 61 % (Wang et al. 2007; Chrispal et al. 2010; Griffith et al. 2014). Other predictors of mortality include the duration of fever, severity of illness on presentation to hospital, elevated serum creatinine (indicative of renal failure), and shock (Varghese et al. 2006; Chrispal et al. 2010; Griffith et al. 2014).

Coinfections of *O. tsutsugamushi* with several other pathogens have been reported. Diseases shown to occur concurrently with scrub typhus include pneumonia caused by *Mycoplasma pneumoniae* (Lee et al. 2015c), murine typhus (Phommasone et al. 2013), Q fever (Lai et al. 2009), leptospirosis (Watt et al. 2003a; Chen et al. 2007; Sonthayanon et al. 2013), chicken pox (Chandramohan et al. 2015), malaria (Mahajan et al. 2014), and dengue (Kumar et al. 2014). Several of the coinfecting pathogens are susceptible to the antimicrobials used to treat scrub typhus. However, a coinfection should be suspected in scrub typhus patients if defervescence does not occur within 48–72 h of appropriate antimicrobial treatment and combination therapy may be required (Wei et al. 2012). Unusually, one *O. tsutsugamushi* coinfection may be advantageous to the patient. In Thailand, researchers observed that HIV-1 patients with scrub typhus experienced a reduction in viral load, and serum from an HIV-1 negative scrub typhus patient had a potent suppressive effect on the virus in vitro (Watt et al. 2000a). Another study was not able to replicate these results in vitro and showed that instead, HIV-1 replication was induced by *O. tsutsugamushi* (Moriuchi et al. 2003). Further work by the Thai researchers demonstrated that antibodies produced in acute scrub typhus suppressed CXCR4-HIV-1 viruses but not those utilizing the CCR5 coreceptor. However, the suppressive effect on the CXCR4-utilizing viruses was potent and long lasting

(Watt et al. 2013). This was the first description of antibodies to one organism having a toxic effect on another infectious agent.

5.2 Diagnosis

The clinical presentation of scrub typhus is notoriously nonspecific. Since the primary manifestation is an acute, undifferentiated fever, when a patient first presents to a healthcare facility there is little to distinguish scrub typhus from other diseases such as typhoid, leptospirosis, and dengue, which are usually endemic in the same areas (Suttinont et al. 2006; Watt et al. 2003b). A recent Korean study developed a prediction rule for identifying suspected cases of scrub typhus in patients with acute undifferentiated fever. The rule comprised five predictors of disease that were derived from a multiple regression model, with each assigned a points value. Predictors were age ≥65 years old (two points), recent history of fieldwork or outdoor activity (one point), onset during the known outbreak period (September to December in Korea; two points), myalgia (one point), and presence of an eschar (two points). Using a cutoff value of four or more points, the sensitivity and specificity of the prediction rule for diagnosis of scrub typhus were 92.7 % and 90.9 %, respectively. While application of this prediction rule does not replace confirmatory testing, it can be used at the time of admission, allowing for the prompt initiation of treatment. However, it should not be used in more complicated cases of scrub typhus with clinical manifestations such as pneumonia or meningitis, and its use outside of Korea has not yet been validated (Jung et al. 2015).

The laboratory and radiographical findings in scrub typhus patients (Table 16.3 and Fig. 16.8) do not provide a definitive diagnosis, although in combination with other symptoms and signs they may lead to a strong suspicion of the disease. For example, in a cohort of Indian patients, a combination of elevated transaminases, thrombocytopenia, and leukocytosis had the best predictive values for diagnosing patients with acute undifferentiated fever (Varghese et al. 2006).

Diagnostic tests for scrub typhus are mostly based on serological methodologies.

The oldest test, the Weil–Felix OXK, is based on the cross-reaction of the OXK antigen from *Proteus mirabilis* with scrub typhus IgM antibodies, resulting in agglutination of the serum (Amano et al. 1992). It has long been recognized that its sensitivity and specificity are poor, particularly in the early stages of *O. tsutsugamushi* infection (Brown et al. 1983; Isaac et al. 2004). For example, in a comparison between the Weil–Felix OXK and indirect immunoperoxidase (IIP) tests, only 10 % of serum samples positive by IIP gave a positive reaction in the Weil–Felix test within the first 9 days of illness (Amano et al. 1992). However, the Weil–Felix test is very cheap and easy to perform, therefore despite its severe limitations, it is still used in some resource-poor regions in which other serological tests are not available. It can be a useful tool in these settings, provided clinicians are aware of its pitfalls and results are interpreted in the correct clinical context (Mahajan et al. 2006).

Table 16.3 Summary of laboratory and radiographical findings in scrub typhus patients

Investigation	Finding	Reported prevalence	Reference
White blood cell count	Leukocytosis Leukocytes >10,000/μL	9–54 %	Ogawa et al. (2002); Mathai et al. (2003); Song et al. (2004); Chrispal et al. (2010); Hu et al. (2015)
Platelet count	Thrombocytopenia Platelets <10,000/μL	21–44 %	Tsay and Chang (1998); Mathai et al. (2003); Song et al. (2004); Chrispal et al. (2010); Griffith et al. (2014); Hu et al. (2015)
	Severe thrombocytopenia Platelets < 50,000/μL	25–47 %	
Serum albumin	Hypoalbuminemia <3.0 g/dL	16–69 %	Song et al. (2004); Kim et al. (2010)
Serum myoglobin	Elevated	35 %	Choi et al. (2000)
Renal function	Elevated serum creatinine > 1.6 mg/dL or > 120 μmol/L	15–37 %	Mathai et al. (2003); Song et al. (2004)
	Elevated blood urea nitrogen > 20 mg/dL	49 %	Jung et al. (2015)
Hepatic function	Elevated transaminases Aspartate transaminase > 60 IU/L Alanine transaminase > 60 IU/L	75–95 %	Chrispal et al. (2010), Song et al. (2004), Ogawa et al. (2002), Mathai et al. (2003), Choi et al. (2000), Zhang et al. (2012), Tsay and Chang (1998)
	Elevated C-reactive protein > 10 mg/dL	25–96 %	Ogawa et al. (2002); Kim et al. (2010); Zhang et al. (2012); Hu et al. (2015)
	Elevated lactate dehydrogenase	92 %	Ogawa et al. (2002); Kim et al. (2010)
	Elevated bilirubin > 1 mg/dL or > 25 μmol/L	18–29 %	Ogawa et al. (2002); Mathai et al. (2003); Kim et al. (2010); Jung et al. (2015)
Partial oxygen pressure of arterial blood (PaO_2)	Hypoxia PaO_2 < 60 mmHg	24–34 %	Song et al. (2004); Chrispal et al. (2010)
Blood pressure	Hypotension Arterial systolic pressure < 90 mmHg	17 %	Song et al. (2004)
Chest x-ray	Normal	50 %	Choi et al. (2000); Mathai et al. (2003); Song et al. (2004); Jeong et al. (2007)
	Pulmonary abnormalities	37–78 %	
	Pleural effusion	42–55 %	
	Hilar enlargement	14–45 %	
	Cardiomegaly	15–38 %	
	Interstitial pneumonia	51 %	Song et al. (2004)

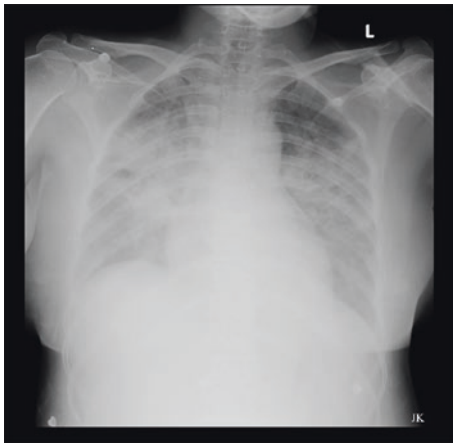

Fig. 16.8 Chest X-ray of a scrub typhus patient. Radiograph shows a diffuse interstitial pneumonia. The patient developed ARDS but responded well to treatment with doxycycline. Image courtesy of Munegowda Koralur

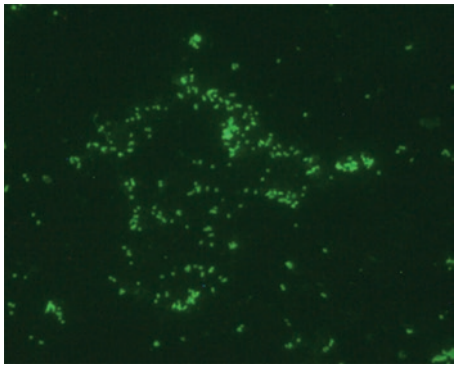

Fig. 16.9 Indirect immunofluorescence assay for scrub typhus diagnosis. Image shows a positive result caused by the binding of antibodies to *O. tsutsugamushi* in the patient serum to antigen from the organism immobilized on the slide. A secondary anti-human antibody labeled with fluorescein isothiocyanate (FITC) enables detection of this reaction by fluorescent microscopy

The currently recognized gold standard test is the indirect immunofluorescence assay (IFA) (Blacksell et al. 2007), in which antibodies to *O. tsutsugamushi* present in the serum of the patient are bound to antigen on a slide, then detected using a fluorescently labeled anti-human antibody (Fig. 16.9).

The most commonly used antigens are a mixture of the Karp, Kato, and Gilliam serotypes (Blacksell et al. 2007), although in some areas, local serotypes are also included (Blacksell et al. 2007; Koh et al. 2010). A positive result is conventionally defined by an IgM titer of ≥1:400 on a single sample taken on admission, or by a fourfold (or greater) rise in titer between paired acute and convalescent samples to ≥1:200 (Brown et al. 1983). However, these conventional cutoff titers may lead to false-positive results, particularly where testing is performed on the admission sam-

ple alone (Blacksell et al. 2007; Lim et al. 2015a). A more recent study proposed revised cutoffs of ≥1:3200 for a single sample, or at least a fourfold rise to ≥1:3200 in the convalescent sample of a pair. These newly proposed optimal cutoffs resulted in an assay sensitivity and specificity of 81 % and 100 %, respectively (Lim et al. 2015a). It is recommended that if IFA is to be used for scrub typhus diagnosis in an endemic country, a local cutoff should be determined based on the scrub typhus seroprevalence rate in the healthy population. This better enables acute infection to be distinguished from previous exposure, which is especially important if testing is performed on only the acute sample (Blacksell et al. 2007). Besides difficulties with determining the appropriate cutoff, the IFA has additional limitations. Since a microscopist reading the slide determines the end-point titer, the test is inherently subjective. Inter- and intraoperator variability has been demonstrated and microscopists must undergo several months of training or be supervised by a more experienced staff member before their results can be considered reliable (Phetsouvanh et al. 2013). The main drawback of the IFA, however, is the relatively high cost and its requirement for a fluorescent microscope. This restricts its use in many of the resource-limited areas in which scrub typhus occurs (Koh et al. 2010). An alternative to the IFA is the IIP, in which the secondary antibody is labeled with peroxidase instead of a fluorochrome (Yamamoto and Minamishima 1982; Kelly et al. 1988). This eliminates the need for a fluorescent microscope, although the method suffers from the same limitations as IFA in terms of cutoff determination.

A scrub typhus IgM ELISA was first developed in 1979, when it was shown to have a similar sensitivity and specificity to the IFA (Dasch et al. 1979). An assay utilizing the *O. tsutsugamushi*-specific recombinant 56-kDa antigen is now available as a commercial kit and more recent studies have demonstrated a similar performance, with sensitivities in the range of 85–93 % and specificities between 94 and 97.5 % (Coleman et al. 2002; Prakash et al. 2006; Koraluru et al. 2015). Although the ELISA does not require specific training or equipment, the cost of the kit may still be too high for some laboratories and its availability is limited in some scrub typhus endemic countries (Isaac et al. 2004).

Numerous assays for the molecular detection of *O. tsutsugamushi* by PCR have been published and used in clinical settings. The most common targets of these assays are the genes encoding the 56-kDa antigen (Furuya et al. 1993; Horinouchi et al. 1996) and 47-kDa surface antigen (Jiang et al. 2004; Singhsilarak et al. 2005), plus the 16S rRNA (Sonthayanon et al. 2006; Kim et al. 2006a, 2016) and *groEL* genes (Park et al. 2005; Paris et al. 2009). PCR is most useful for diagnosis in the early stage of scrub typhus, before antibodies are detectable by serological methods. During this stage of the disease, PCR has been demonstrated to be more sensitive than IFA. In a Korean study, a positive result was obtained by nested PCR on the buffy coat of 19 out of 22 scrub typhus patients that were all negative by IFA on admission (Kim et al. 2006a) and similar results were obtained in a Thai study (Manosroi et al. 2003). The sensitivity of PCR in these two small studies of 21 and 118 patients was reportedly high (82.2 and 90.5 %) (Manosroi et al. 2003; Kim et al. 2006a). However, in larger studies of more than 180 patients, the diagnostic sensi-

tivities of PCRs targeting the 16S rRNA gene, 56-kDa antigen gene, and 47-kDa antigen gene were only 44.8 %, 29 %, and 28.6 %, respectively (Sonthayanon et al. 2006; Watthanaworawit et al. 2013). Conflicting results have also been obtained from studies in which various PCR assays were compared. One demonstrated a real-time PCR targeting the 47-kDa antigen gene to be more sensitive than nested and conventional assays with the same target (Kim et al. 2011a). However, a more recent study showed that a conventional assay targeting the 16S rRNA gene was the most sensitive compared with conventional PCRs targeting different genes, nested and real-time assays (Kim et al. 2016). PCR sensitivity is likely influenced by several factors. Due to the sequence variability between different *O. tsutsugamushi* serotypes, particularly within the 56-kDa antigen gene, primers may not anneal as efficiently to some strains and may not even detect new serotypes. Detection may be more successful if PCR is performed on DNA extracted from the buffy coat of the blood rather than the whole EDTA blood sample, as the bacterial load has been shown to be approximately tenfold higher in the cellular fraction (Paris et al. 2008). Antibiotics have been shown to reduce the sensitivity of PCR detection of *O. tsutsugamushi* in the blood to 10 % by the fourth day after initiation of treatment (Kim and Byun 2008). Therefore, the assay should ideally be performed prior to antibiotic administration or at least only within 3 days after treatment initiation. Alternatively, PCR can be performed on eschars (Lee et al. 2006; Kim et al. 2006b). Detection of bacterial DNA from an eschar is less affected by antibiotic treatment (Kim et al. 2006b), but this sample is not available from all scrub typhus patients. Similarly to the IFA, the major disadvantage of PCR as a diagnostic method is its requirement for expensive, specialized equipment and staff training, which is not available in many scrub typhus endemic areas.

Besides serological and molecular diagnostic techniques, lesser used methods include immunohistochemical staining of the eschar (Kim et al. 2008) and isolation of *O. tsutsugamushi*, either in cell culture (Luksameetanasan et al. 2007) or by inoculation into a mouse (Casleton et al. 1998). Since the latter must be performed in a BSL-3 laboratory, it is not attempted routinely. Growth of the organism is often slow; therefore, results are of little use to the patient and treating clinician. However, it is important to obtain isolates of the bacteria to identify new serotypes and to allow further studies to be performed.

The common theme in scrub typhus diagnostics is the lack of resources in many areas in which the disease occurs. For this reason, there has been a focus in recent years on developing rapid, point-of-care tests that could provide an early, accurate diagnosis in these resource-limited settings. An alternative method to traditional PCR for the molecular detection of *O. tsutsugamushi* is the loop-mediated isothermal PCR assay (LAMP) (Paris et al. 2008, 2011). This method utilizes six primers and can amplify from a few copies to 10^9 copies in less than an hour. Amplification takes place at a single temperature (65 °C) and a positive result is determined by naked eye inspection of the reaction tube to visualize turbidity or a pellet (Notomi et al. 2000), therefore the only equipment required is a water bath or heat block. In an acute disease setting, LAMP was shown to have a diagnostic sensitivity of 53 % and specificity of 94 % (Paris et al. 2011).

Several immunochromatographic tests (ICTs) have been developed to provide point-of-care serological testing for scrub typhus, and some of these are now commercially available. In recent studies, while the specificity of these tests ranged between 68 and 95 %, sensitivity was lower at only 23–68 % (Blacksell et al. 2010a, b, 2012; Watthanaworawit et al. 2015). Diagnostic sensitivity was improved by 10–20 % if an ICT was combined with LAMP (Paris et al. 2011; Blacksell et al. 2012), but further research is required before a point-of-care scrub typhus test can be widely implemented. Most recently, publications have described a dot-ELISA (Rodkvamtook et al. 2015) and another ICT (Kingston et al. 2015), both based on recombinant 56-kDa antigens. Previously, a panel of scrub typhus infection criteria (STIC) was proposed for confirmation of positive cases, which could then be used in the evaluation of new diagnostic methods (Paris et al. 2011). A sample had to satisfy one of the four criteria, which were as follows: (1) positive cell culture isolation of *O. tsutsugamushi*; (2) an admission IgM titer ≥ 1:12,800; (3) a fourfold rising IgM titer in paired serum samples; and (4) a positive result in at least two out of three PCR assays. Using STIC as a reference standard assumes that it is 100 % specific and sensitive, when in fact this may not be the case. A recent reanalysis of data from 161 patients using Bayesian latent class models gave different results for the sensitivities and specificities of various diagnostic tests compared to when STIC was used as the reference standard (Lim et al. 2015b). It is now recommended that any new tests for scrub typhus diagnosis be evaluated against a carefully selected set of existing tests using appropriate statistical models (Lim et al. 2015b).

5.3 Treatment and Control

Scrub typhus is readily curable using timely, appropriate antibiotics. Chloramphenicol was the first antibiotic shown to be effective at treating the disease (Smadel et al. 1949), but it was subsequently shown that fever and other symptoms were eliminated more rapidly using tetracycline (Sheehy et al. 1973). The mainstay of scrub typhus treatment is now doxycycline. Defervescence in the patient occurs so promptly (24–36 h) after initial administration of the drug that in some areas with limited access to diagnostic testing, this response to treatment is considered diagnostic for scrub typhus (Watt et al. 1996). The reduction in fever correlates with a reduction in production of inflammatory cytokines such as TNF-α, IL-1β, and IL-6, which are up-regulated in scrub typhus patients (Chung et al. 2008). While short treatment regimens of a single dose (Brown et al. 1978b) or 3 days of doxycycline have been demonstrated to be effective (Song et al. 1995), caution should be exhibited if this treatment is initiated within the first 5 days of disease onset. Since the antibiotics shown to be effective against *O. tsutsugamushi* are bacteriostatic rather than bactericidal, they slow the bacterial growth while the patient mounts an immune response to the infection. Thus, there have been cases of patients relapsing when treatment was terminated too early, before the development of antibodies (Smadel et al. 1950; Olson et al. 1981; Im et al. 2014). Doxycycline failure was also observed in a patient who was administered antacids for the gastrointestinal symptoms of

scrub typhus in addition to the antibiotic. The patient developed scrub typhus meningoencephalitis during the course of the treatment, believed to have been caused by the antacids reducing the doxycycline concentration in the serum to subtherapeutic levels (Kim et al. 2011b). Delayed fever resolution in some patients treated with doxycycline in northern Thailand led to the discovery of *O. tsutsugamushi* strains that were resistant to both doxycycline and chloramphenicol (Watt et al. 1996). The driver for this resistance is unclear, although it was postulated that antibiotics may have reached the chigger reservoir via rats feeding on animal food (Rosenberg 1997).

Alternative antibiotics for the treatment of scrub typhus include rifampin (Im et al. 2014; Watt et al. 2000b) and the macrolides azithromycin, telithromycin (Kim et al. 2007b), and roxithromycin (Lee et al. 2003). Rifampin was shown to reduce fever more rapidly than doxycycline (Watt et al. 2000b), although its efficacy in cases of severe scrub typhus has not been evaluated. Azithromycin has been shown to be as effective as doxycycline for treatment of both mild to moderate and severe scrub typhus (Kim et al. 2004b; Jang et al. 2014). Its use is recommended for the treatment of scrub typhus in pregnant women and children under 8 years old, as doxycycline use in these groups is contraindicated due to association of the drug with fetal risk and permanent discoloration of the teeth (Kim et al. 2006c; Cross et al. 2015). However, evidence for these risks was based on tetracycline rather than the newer, semisynthetic doxycycline and there are calls for the contraindication of doxycycline to be reevaluated (Cross et al. 2015). Conflicting reports are available regarding the efficacy of the fluoroquinolones against *O. tsutsugamushi*. One study showed that levofloxacin was an effective treatment for scrub typhus, although patients took longer to become afebrile compared to those administered tetracycline antibiotics (Tsai et al. 2010). Other studies showed that *O. tsutsugamushi* strains were resistant to ciprofloxacin and ofloxacin, and demonstrated the presence of a mutation (Ser83Leu) associated with resistance in the quinolone resistance-determining region of the *gyrA* gene (Tantibhedhyangkul et al. 2010; Jang et al. 2013). Therefore, the use of fluoroquinolones is not advocated.

In the case of coinfections with *O. tsutsugamushi* and another pathogen, combination therapy may be required for the effective resolution of both diseases. For example, the recommended treatment for a patient with concurrent scrub typhus and leptospirosis is ceftriaxone and doxycycline (Lee et al. 2014). In cases of severe scrub typhus pneumonia and ARDS, empirical treatment with a beta-lactam antibiotic with doxycycline or a macrolide is sometimes used (Lee et al. 2014). This practice may need to be revised in light of the finding that the efficacies of doxycycline, azithromycin, and rifampin against *O. tsutsugamushi* are lessened in the presence of the beta-lactam cefotaxime (Lee et al. 2014). In contrast, the antimalarial agent chloroquine has been shown to increase the antimicrobial effect of doxycycline against *O. tsutsugamushi* in vitro (Son and Chung 2014).

Although most patients make a full recovery from scrub typhus, there is evidence that *O. tsutsugamushi* is not completely cleared and may persist in the body. In an early study, the organism was isolated from an asymptomatic patient who had confirmed scrub typhus infection more than 1 year prior (Smadel et al. 1952). More recently, *O. tsutsugamushi* was isolated from six recovered patients, up to 18

months after disease onset (Chung et al. 2012). The significance of persistent infection is not known but it is possible that recrudescence may occur after primary infection (Im et al. 2014).

Despite extensive research, there is currently no vaccine available to prevent scrub typhus. Chemoprophylaxis of a 200 mg weekly dose of doxycycline has been demonstrated to be effective at preventing the disease and is recommended for people entering a scrub typhus endemic region where they are likely to have a high risk of exposure to mites (Olson et al. 1980; Twartz et al. 1982). Generally however, control of scrub typhus relies on measures to reduce the risk of chigger bites. Toward the end of World War II, military personnel in scrub typhus endemic areas were encouraged to treat their uniforms with the mosquito repellent dimethylphthalate, which was also effective against chiggers. The Australian Army introduced dibutylphthalate to its soldiers as "antimite fluid," which was more resistant to washing (Philip 1948; Frances 2011), and a 90 % reduction in scrub typhus cases was achieved through its use (Mcculloch 1946). It is now recommended that military uniforms be treated with permethrin (Frances et al. 1992; Frances 2011) or dibutylphthalate (Hengbin et al. 2006) to reduce chigger (and other arthropod) attachment.

Local health authorities in endemic areas have implemented education programs and proposed recommendations to civilian populations in order to reduce cases of scrub typhus. Preventative measures include wearing protective clothing such as arm warmers or gumboots during work activities, washing after work, changing into a different set of clothes to sleep, and using insect repellent (Sharma et al. 2009; Kim et al. 2012). The commonly used insect repellent, DEET (*N*-*N*-diethyl-*m*-toluamide), is effective against chiggers (Buescher et al. 1984) and recent studies have shown some essential oils from plant species, particularly *Syzygium aromaticum* (clove) and *Melaleuca alternifolia* (tea tree) have a chigger repellent effect. These may be cheaper and less harmful than synthetic chemicals (Eamsobhana et al. 2009; Rodkvamtook et al. 2012). A scrub typhus prevention program implemented in several public health centers in Korea was attributed to a decline in the reported cases of disease. A cost–benefit analysis estimated it saved $6.66 million each year by reducing medical costs and productivity losses (Kim et al. 2012), therefore similar programs may be beneficial in other endemic areas. In addition to personal protective measures, steps can be taken to control the environment and rodent populations, making conditions less favorable for mites. These include the trapping or poisoning of rodents, regular cleaning of buildings inside and out, management of human and food waste, and maintenance of the natural habitat such as preventing weed growth around buildings (Hengbin et al. 2006).

6 Conclusions

Scrub typhus caused by *O. tsutsugamushi* remains a serious and potentially life-threatening disease. Despite years of research, there are still many unresolved issues relating to disease ecology, epidemiology, pathogenesis, diagnosis, and immunity

(Paris et al. 2013). Work continues to address these problems and increase our understanding of the disease and its causative agent.

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Chapter 17
Epidemiological Trends of Scrub Typhus: Global Incidence and Vector Distribution

Na-Young Ha, Gwanghun Kim, and Nam-Hyuk Cho

Scrub typhus is an acute febrile illness caused by infection with an obligate intracellular bacterium, *Orientia tsutsugamushi* (Tamura et al. 1995). The causative agent is transmitted to humans by infected larval trombiculid mites during the feeding process and is maintained in nature through vector mites transovarially as well as transstadially (Traub and Wisseman 1974). Clinical symptoms of infected patients can vary from mild to lethal. Early clinical manifestations are eschar, fever, headache, myalgia, and rash, making it difficult to differentiate scrub typhus from other acute febrile infections. Delayed diagnosis and treatment with proper antibiotics often leads to acute respiratory distress, renal failure, meningoencephalitis, and multiple organ failure (Paris et al. 2013). The mortality rate of scrub typhus is quite variable with a median mortality of approximately 6.0 % (Taylor et al. 2015), but reaches up to 70 % in untreated patients (Kawamura et al. 1995).

The striking feature of scrub typhus is an endemicity within Asia, northern Australia, and the islands in western Pacific (Kelly et al. 2009). Considering that clinical features of scrub typhus were described in an ancient Chinese document

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from 313 A.D. and in Japanese modern literature from the early 1800s (Kelly et al. 2002), it appears that the disease has long been confined to its endemic area. There have been some exceptions, with several cases of suspected scrub typhus reported outside of the endemic region (Balcells et al. 2011; Ghorbani et al. 1997) and one new species, *O. chuto*, was identified in Dubai (Izzard et al. 2010). These indicate a wider geographic distribution and genetic diversity of the genus, but the majority of scrub typhus cases have been reported within the endemic region where the vector mites are found. Therefore, the ecology of the vector mite is the primary factor that determines the epidemiological features of scrub typhus (Kelly et al. 2009). Another emerging issue of scrub typhus is the increasing burden on public health within endemic regions. Scrub typhus is probably one of the most underdiagnosed and underreported febrile illness requiring hospitalization in endemic regions (Paris et al. 2013). It is estimated that more than a million cases occur annually and a billion people are exposed to scrub typhus within the highly populated endemic regions (Watt and Parola 2003). In fact, scrub typhus accounts for up to 20 % of febrile hospital admissions in rural areas of southern Asia (Brown et al. 1977; Kasper et al. 2012; Phongmany et al. 2006; Varghese et al. 2006). In addition, its new emergence in the northern China (Zhang et al. 2010), rapid increase in South Korea (Jeong et al. 2013), and continuous outbreaks in several countries (Lewis et al. 2003; Rodkvamtook et al. 2011; Sethi et al. 2014) have recently drawn much attention from the public health agencies in endemic countries. Yet, there has been little effort to update epidemiological data on the precise disease burden and the map of endemicity in the global level in the last several decades (Kelly et al. 2009). Given this, the need for information on the environmental factors that affect the epidemiology of scrub typhus continues to grow as countries seek proper measures to combat reemerging and/or rising incidence in the endemic countries.

In this review, we collected epidemiological data on vector mites and scrub typhus cases from published papers, text books, and surveillance databases provided by public health agencies in several countries summarize the current global epidemiological trends of scrub typhus.

1 Epidemiological Trends and Spatiotemporal Pattern of Scrub Typhus at the Global Level

In order to investigate the epidemiological trends of scrub typhus at the global level, we collected incidence data from disease surveillance systems managed by public health agencies in several countries, published papers, and commercial databases (Table 17.1). Since each country uses different diagnosis standards for disease confirmation, we defined confirmed scrub typhus as positive specific serum IgG or IgM by indirect immunofluorescence assay, detection of *O. tsutsugamushi* DNA by PCR, or isolation of *O. tsutsugamushi* from clinical specimens. All other methods (e.g., cross-reactive Weil-Felix test or diagnosis based on clinical manifestations) were regarded as clinically reported cases. As shown in Fig. 17.1, annual cases of

Table 17.1 Data resources for the epidemiological study on scrub typhus

Country	Resources and references
China	PLoS Negl Trop Dis. 2013 7(12):e2493.
Hong Kong	Travel Med Infect Dis. 2011 9(3):95–105
India	Front. Public Health, 2014 2:00168
Japan	http://idsc.nih.go.jp/idwr/ J Epidemiol 2007 Suppl:S48–55
Oceania	Acta Trop. 2014 pii: S0001-706X(14)00334-9
Sri Lanka	Emerg Infect Dis. 2012 18(5):825-9 Int J Infect Dis. 2013 17(11):e988-92
South Korea	http://is.cdc.go.kr/nstst/
Taiwan	http://nidss.cdc.gov.tw/
Thailand	http://203.157.15.110/nphss/
Others	Australo-Asian Spotted Fevers: Global Status—2013 edition. Infectious Disease of China—2013 edition Infectious Disease of India—2013 edition GIDEON Informatics, Inc. (http://www.gideononline.com/)

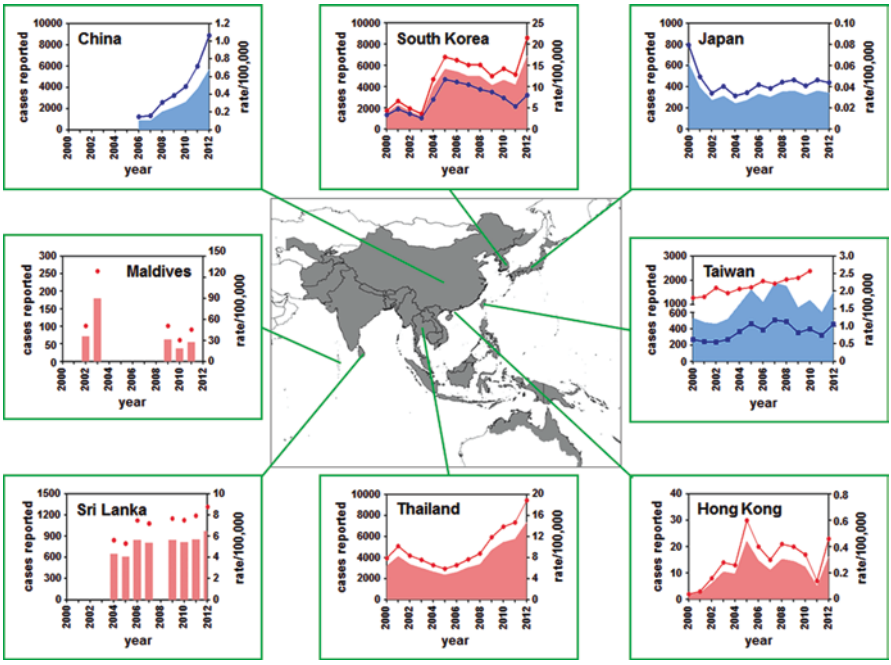

Fig. 17.1 Geographical distribution and the trends of annual incidences in endemic countries

scrub typhus have gradually increased in the most of the countries that had a well-managed surveillance system for scrub typhus during the last decade. In particular, there has been a noticeably rapid increase in incidence in China (Zhang et al. 2013b) and South Korea (Jeong et al. 2013) among the countries of endemicity. In contrast, the confirmed cases in Japan curiously and gradually decreased from the 1980s to early 2000s and have persisted at around 400 cases per year thereafter (Berger 2013). Sporadic outbreaks of scrub typhus have been continuously reported in many countries, including India, Maldives, Sri Lanka, and Oceania (Berger 2013; Derne et al. 2014). The current incidence rate per 100,000 population ranges from ~ 0.04 in Japan to ~ 15.0 in South Korea (Fig. 17.1). Although we should be cautious in translating and comparing scrub typhus epidemiological data between endemic countries because of differences in standard methods of diagnosis and surveillance and reporting systems, general trends of increased emergence and incidence within the endemic region are quite obvious and might be partly due to increased awareness and improved diagnostic testing (Paris et al. 2013). The reasons for emergence and reemergence and the true incidence of this neglected disease are the critical issues that need to be investigated (Paris et al. 2013). It may be that the epidemiological characteristics of scrub typhus are attributable to the habitats and changing ecology of the vector mites (Traub and Wisseman 1974).

Seasonal prevalence of scrub typhus is also quite variable among the countries (Fig. 17.2). In the northern parts of endemic regions including South Korea (Kweon et al. 2009) and northern China (Zhang et al. 2012), incidence rate is remarkably seasonal (from autumn to winter, peaking at October to November), whereas disease occurrence is increasingly year-round the closer countries are to tropical regions. In Taiwan (Huang et al. 2012), Hong Kong (Ma et al. 2011), and southern China (Wei et al. 2014), scrub typhus is persistently reported from late spring to winter. Occurrence is the most nearly year-round in Thailand where scrub typhus has a broad peak season from summer to autumn (Fig. 17.2). Scrub typhus in Sri Lanka also presents sporadically throughout the year without any seasonal preponderance (Kularatne et al. 2013). In Japan, occurrence of scrub typhus shows a bimodal distribution with a minor peak in the spring (May and June) and a major peak from fall to winter (October to January) (Hashimoto et al. 2007). It is also interesting to note that seasonal prevalence and the number of cases rapidly changed during the late 1970s and early 1980s in Japan (Ebisawa 1995). For example, scrub typhus in Japan was prevalent during the summer, peaking in August before the 1970s (Ebisawa 1995). This changed in the early 1980s to bimodal peaks in spring and fall (Ebisawa 1995). In addition, the number of scrub typhus cases rapidly increased during the 1980s and reached a maximum peak in 1990 (from 31 cases in 1976 to 941 cases in 1990), before gradually declining thereafter until the early 2000s (313 cases in 2004) (Berger 2013; Ebisawa 1995). Although the rapid changes in epidemiology in the 1980s have not been fully explained, it appears to be vector mite specific (Ebisawa 1995). Based on vector ecology, it has been proposed that classical scrub typhus transmitted by *L. akamushi*, which had a peak incidence in August, was replaced by new types transmitted by *L. palidum* and *L. scutellare* with different peak activity seasons, in spring and fall, respectively, in the late 1970s (Ebisawa 1995).

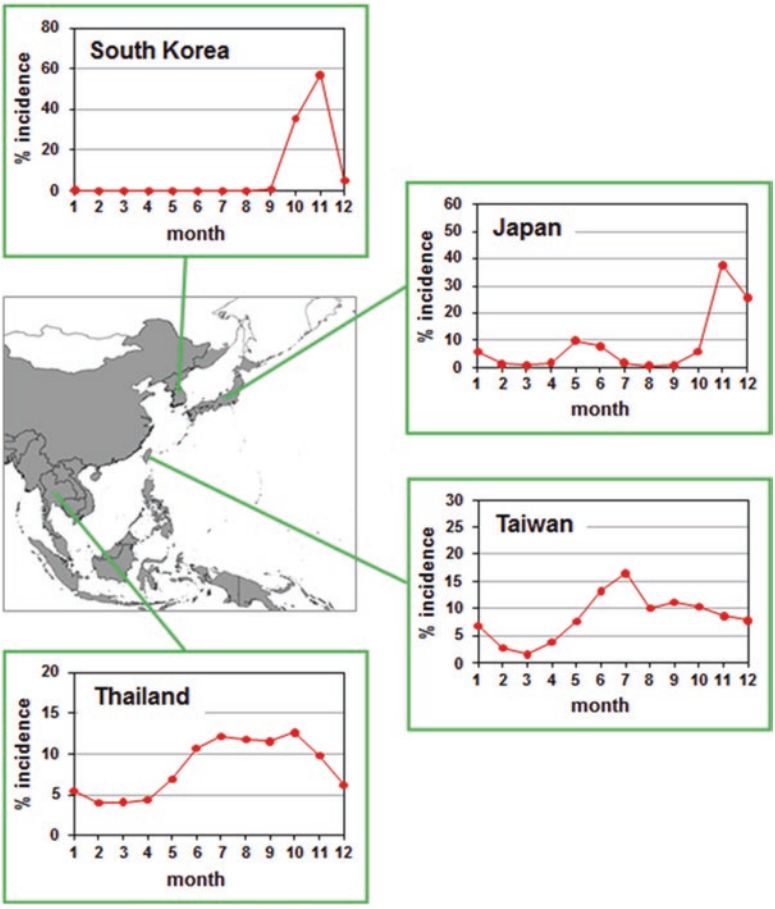

Fig. 17.2 Seasonal prevalence of scrub typhus in several endemic countries

2 Global Distribution of Mite Vectors for Scrub Typhus: Updated Vector Map

Although the distribution of vector mites is the obvious factor affecting the spatio-temporal differences and changes in the epidemiology of scrub typhus, current epidemiological map of the vector species in the global level is either roughly outlined in the country level or based on the data obtained during the last century (Kawamura et al. 1995; Kelly et al. 2009; Traub and Wisseman 1974). The global vector map currently available was published by World Health Organization in 1989 (W.H.O 1989) and was based on data from before 1974 (Traub and Wisseman 1974). Therefore, it does not accurately reflect current scrub typhus status and has to be updated. We searched references for epidemiology data as well as information on

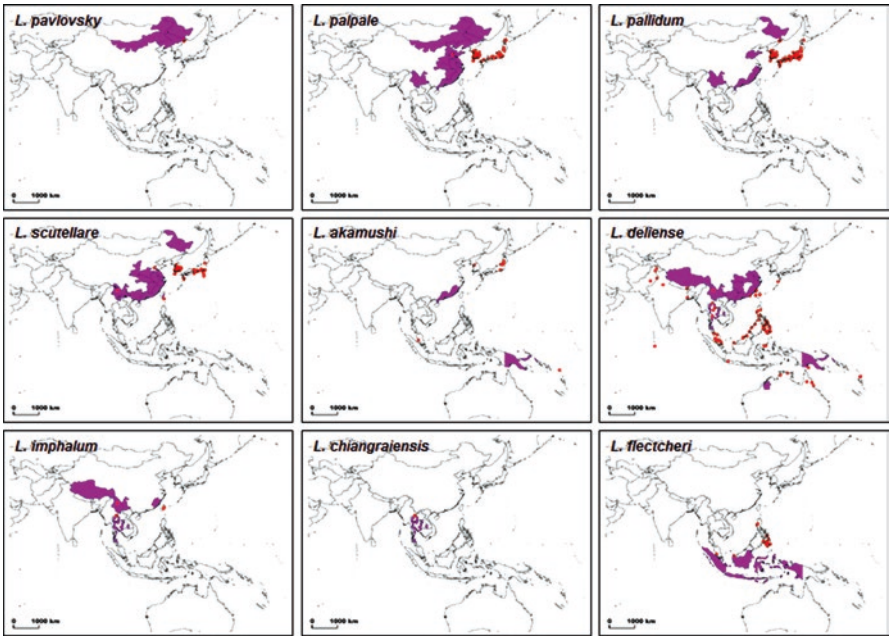

Fig. 17.3 Vector map of scrub typhus. Geographical distributions of nine representative *Leptotrombidium* species are presented. If the collection sites of vector identification were specified, the sites are indicated as *red dots*; otherwise, the collection sites are presented in province level

the distribution of *Leptotrombidium*, the primary species responsible for the transmission of scrub typhus (until Dec. 2014) and constructed an updated vector map within the endemic region (Fig. 17.3). Several other trombiculid mites are not included in the map, either because of their relative rarity or because they are unproven human vectors (Kawamura et al. 1995; Traub and Wisseman 1974). However, it should be noted that many other species of trombiculid mites carrying *O. tsutsugamushi* have been continuously reported in specific parts of the endemic region (Lee et al. 2011; Park et al. 2015; Zhang et al. 2013a).

There are several notable characteristics in the ecology and distribution of *Leptotrombidium* species within the endemic region (Fig. 17.3). Three major *Leptotrombidium* species, *L. palpale*, *L. pallidum*, and *L. scutellare*, prevalent in the northeastern area (i.e., South Korea and Japan) of endemicity (Ebisawa 1995; Roh et al. 2014; W.H.O 1989) have been recently found in a broader range of area including eastern China (Zhan et al. 2013; Zhang et al. 2013a). In particular, *L. scutellare*, the agent of autumn–winter scrub typhus, is now the primary vector and reservoir in northern China (Zhang et al. 2010, 2013b), South Korea (Roh et al. 2014), and Japan (Ebisawa 1995). It is also notable that, *L. pavlovskyi*, the primary vector responsible for scrub typhus in the Primorye region of Russia, the northern limit of

endemicity, during the 1960s was also prevalent in the 1990s, but the pathogen could not be isolated from the vectors (Urakami et al. 1999). This indicates that the prevalence of scrub typhus in the Primorye region has decreased considerably, even though there has been no updated data available thereafter.

L. delicense is the primary vector in the southern parts of the endemic region, ranging from southern China and Taiwan to the north, Pakistan to the west, and northern Australia and western Pacific islands to the south. However, there has been local variation of major vectors such as *L. scutellare* in Yunnan province, southern China (Zhan et al. 2013), *L. imphalum* in eastern Taiwan (Kuo et al. 2011), *L. chiangraiensis* and *L. imphalum* in northern Thailand (Tanskul et al. 1998). *L. fletcheri* and *L. arenicola* were reported by several countries in southern Asia near the tropic region before 1990 (Kelly et al. 2009). From a global view on the distribution of the primary vector species, it seems like that their localization is largely restricted by the climate factors, temperature, and precipitation (Traub and Wisseman 1974), which may confine the disease within the "Tsutsugamushi triangle" by acting as limiting factors for the colonization of the mites, i.e., the low temperature and precipitation in the northern boundary and the low precipitation in the western boundary. Primary vectors for northern scrub typhus in the endemic region, *L. palpale, L. pallidum*, and *L. scutellare*, are distributed in a temperate area with annual average temperature range approximately 0–21 °C and with an average precipitation of over 500 mm/year. *L. delicense*, the most prevalent vector in the southern area, is localized in subtropical and tropical regions with an average temperature range of approximately 15–30 °C and with an average precipitation of over 1000 mm/year. *L. imphalum* and *L. fletcheri* are also confined to subtropical and tropical areas, respectively. It should also be noted that the dominant species of chigger mites in local areas can vary across different geographic regions of varying altitudes, seasonal variation, and changes in habitat ecology natural and man-made (Kuo et al. 2011; Traub and Wisseman 1974; Zhan et al. 2013). In fact, an interesting phenomenon observed during our review of references on vector distribution is that local changes in the distribution of vector species in the endemic region have been often reported. The disappearance of *L. akamushi* in Japan during the 1960s and emergence of *L. palidum* and *L. scutellare* from the late 1970s that resulted in changes in seasonal prevalence, as mentioned above, is the representative example (Ebisawa 1995). North-bound expansion of *L. scutellare* observed in South Korea during the last two decades is also correlated with the rapid increase in scrub typhus incidence, especially during autumn (Roh et al. 2014). An increase in the relative fraction of *L. imphalum* on the main island of Taiwan might be responsible for the emergence of scrub typhus during the recent decades, whereas *L. deliense* has dominating the surrounding islands of Taiwan (Kuo et al. 2011). Local changes of *L. deliense* to *L. scutellare* as the primary vector were also reported in the Yunnan province of southern China (Zhan et al. 2013) although it is not clear whether this change has affected the local epidemiology of scrub typhus.

Another striking phenomenon is the broad distribution of two primary vectors responsible endemic scrub typhus, i.e., *L. scutellare* in the northern area and *L. delicense* in the southern area. When compared to the previous vector map from

WHO (1989), *L. delicense* has occupied the tropical and subtropical area for a long time (Traub and Wisseman 1974), whereas *L. scutellare* has expanded in the last several decades. Both the north-bound expansion of *L. scutellare* in mainland China (Zhang et al. 2010, 2013b) and South Korea (Roh et al. 2014), and the south-bound in southern China (Zhan et al. 2013) and Taiwan (Kuo et al. 2011; Wang et al. 2004) have been reported recently. As a result, the southern area of mainland China and Taiwan have become mixing grounds of the two primary vector species nowadays. It is also notable that *L. imphalum*, the primary vector in northern Thailand (Tanskul et al. 1998), has also been reported as the dominant mite in a local area of Taiwan (Kuo et al. 2011) as mentioned above, such that diverse vector species prevail in northern and southern Taiwan islands.

3 Potential Role of Migratory Birds in Spread of Scrub Typhus

In addition to the widespread of major vectors and diverse genotypes of *O. tsutsugamushi* over the endemic region including many islands in the Indian and Pacific Oceans (Kelly et al. 2009), continuous fluctuation in the distribution of chigger mites at the local level suggest that birds may be potential transporters of infected mites (Kawamura et al. 1995; Traub and Wisseman 1974). Because larval mites apparently do not travel more than a few meters from where they hatch and usually form "mite islands" ranging from a few centimeters to meters (Traub and Wisseman 1974), their ability to migrate on their own is very limited and their movement is mainly associated with the migration of hosts infested with chigger mites (Kawamura et al. 1995). However, the activity range of field rodents, the principle parasitized host of trombiculid mites, is also ecologically limited (Kawamura et al. 1995). Therefore, birds have been considered to be the primary phoretic hosts, spreading the trombiculid mites to distant locations or to other islands (Kawamura et al. 1995; Nadchatram 2008; Traub and Wisseman 1974). Since the early 1900s, dozens of studies have reported chigger mites feeding on diverse species of domestic or migratory birds (Nadchatram 2008; Traub and Wisseman 1974; Yoshino et al. 2011). Considering that chigger mites attach and feed on host animals for about 36–72 h and withstand harsh environmental condition such as temperatures of −20 °C for a period of several weeks (Traub and Wisseman 1968), they can travel hundreds to thousands of kilometers while attached to migratory birds, before landing in a new geographic area that they may colonize if environmental conditions are optimal for their survival (Tsiodras et al. 2008). It has been proposed that wild and migratory birds may have played a significant role in the peridomestic or international transmission of diverse infectious agents including Avian influenza and Lyme disease by acting as biological carriers or mechanical carriers (Fuller et al. 2012; Hubalek 2004). Since *O. tsutsugamushi* has rarely been recovered from wild birds (Kitaoka et al. 1976; Traub and Wisseman 1974), they are more likely mechanical carriers for

short- or long-distance transmission of scrub typhus rather than biological carriers. Therefore, consideration of avian migration patterns might be useful in understanding and predicting epidemiological changes, such as local outbreaks of scrub typhus and spread of mite vectors as well as *O. tsutsugamushi* genotypes.

4 Conclusion

Scrub typhus is an ancient infectious disease transmitted through the bites of infected chiggers in the Asian-Pacific region. Increase in disease burden and widespread of the infected vector species has been reported throughout the endemic region. Furthermore, recent reports of urbanization of scrub typhus suggest that this disease may have a significant impact on public health issues (Maude et al. 2014; Park et al. 2015). Environmental and social changes driven by predicted climate changes may also lead to the emergence of rickettsioses in the endemic region (Derne et al. 2015). Therefore, continuous surveillance of the geographical distribution and the changing ecology of the vector mites as well as disease incidence are important for the proper control of scrub typhus.

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Chapter 18
Biology of *Orientia tsutsugamushi*

Rahul Narang

Orientia tsutsugamushi previously called *Rickettsia tsutsugamushi* is a small, intracellular, Gram-negative bacterium belonging to Order Rickettsiales and is the causative agent of tsutsugamushi disease commonly known as scrub typhus.

1 History

History of scrub typhus can be traced back to 313 A.D. when it was first described in Chinese writings and later to early 1800 when it was described in Japan (Kelly et al. 2009). First report of this disease was published by Nagayo et al. (1915) and in 1916, Weil Felix test for diagnosis was established (Cruikshank 1927). The organism causing this disease was initially considered to be a Protozoan and the name of *Theileria tsutsugamushi* was proposed by Hayashi (1920). The organism was initially isolated by Nagayo et al. who proposed the name *Rickettsia orientalis* (Nagayo et al. 1930). The role of chiggers in its transmission was described by Kawarimura (Rehacek and Tarasevich 1988). Ogata (1931) proposed the name *Rickettsia tsutsugamushi* which was accepted by *Bergey's Manual of Determinative Bacteriology* 6th Edition in 1948.

Until 1995 *Orientia* was included in the genus *Rickettsia* as the organisms were obligate intracellular parasites, morphologically similar to Gram-negative bacteria, survived in both vertebrate and arthropod hosts and human infection was mediated by arthropods. However, it was later found that for organisms causing scrub typhus, there existed certain differences from other organisms causing spotted fever and

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typhus fever. The outer leaflet of the cell wall of *Orientia tsutsugamushi* is considerably thicker than the inner leaflet, while the opposite is true for other rickettsial species. In addition, there are differences in constitution of cell wall and thus susceptibility to penicillin. Based on these differences and considering that the causative agent of scrub typhus was discovered by several Japanese workers, it was renamed *Orientia tsutsugamushi* (Tamura et al. 1995). In 2010, a novel species belonging to genus *Orientia* was proposed as "*Orientia chuto*" and the prototype strain of this species was called strain Dubai (Izzard et al. 2010).

2 Habitat and Epidemiology

The disease occurs widely in the Palaearctic, Oriental, and Australasian regions, including south-east Asia where it can account for 10–19 % of patients admitted to hospital with acute undifferentiated fever.

Scrub typhus is transmitted by several species of larval trombiculid mites, which are commonly known as chiggers. Mites are unique among vectors in that they are parasitic in only larval stage. They normally attach to and feed upon only a single vertebrate and therefore cannot acquire an infection from one host and subsequently transmit to a second host (Traub et al. 1975). Transovarial transmission is thought to be the only mechanism for maintenance of *O. tsutsugamushi* in the vector. Infected chiggers can therefore be considered as true host of *O. tsutsugamushi*, and even commonly infected mammals may be dead-end hosts rather than true reservoirs. Frances et al. (2000) demonstrated that *O. tsutsugamushi* could be transmitted to co-feeding mites, and Takahashi et al. (1990) were able to infect chiggers fed on wild rodents; however, neither study determined if infected mites transmitted the rickettsiae to their eggs. Traub et al. (1975) documented horizontal transmission of *O. tsutsugamushi* although they discussed the possibility that the observation was not representative of natural transmission.

Thus the role of mammals as reservoir of *O. tsutsugamushi* remains a controversial issue. Although various studies have demonstrated that chiggers can acquire *O. tsutsugamushi* during feeding process, to date mammals have not been shown to play a conclusive role in the cyclical transmission of this pathogen (Traub et al. 1975; Walker et al. 1975; Takahashi et al. 1990; Frances et al. 2000).

Foci of infection termed "typhus islands" have been reported in some areas and described as sharply localized and irregularly scattered areas where transmission is common. The sizes of these foci are determined by the range of vector mites and their maintaining hosts (Coleman et al. 2003). Rodents may play a key role in the epidemiology of scrub typhus, as they serve as maintenance hosts for the vector mite, and chigger distribution often directly reflects the distribution of the rodent host. In a study (Coleman et al. 2003) conducted in Thailand on occurrence of scrub typhus in small mammals, the authors found *O. tsutsugamushi* in 10 of the 22 species of the mammals studied. Out of the total, 98 % (553 in 565) mammals belonged to *R. rattus*, *R. locea,* and *B. indica*. As per the authors of that study infection rates

in different species of mammals may be independent of chigger densities. *R. rattus* has been found to maintain *O. tsutsugamushi* infection for months or longer. The question remains whether a particular species of rodent with its associated chiggers could conceivably start a focus of scrub typhus in a new area. Epidemiological studies with species of rodents most likely to get exported to other parts of the world might be the most appropriate means of determining whether their invasion of new areas would expand the range of scrub typhus (Coleman et al. 2003).

3 Microbiology

O. tsutsugamushi are short Gram-negative rods, 0.5–0.8 μm in diameter and 1.2–3.0 μm long. Individual bacterial cell is surrounded by cell wall and cell membrane. Components of typical bacterial cell wall viz. muramic acid, glucosamine, 2-keto 3-deoxyclonic acid, and hydroxyl fatty acids are not present in *Orientia* cell wall, suggesting that these cells lack peptidoglycan and lipopolysaccharide. The outer leaflet of cell wall is considerably thicker than the inner leaflet. The cells do not have flagella, capsule or slime layer, and endospores are not formed. They can be stained with Giemsa or Gimenez stain. In intracellular state, the outer leaflet of cell wall adheres tenaciously to host membrane and host components are not readily removed by the methods used for other *Rickettsia* (Tamura et al. 1995).

In *O. tsutsugamushi*, a 54- to 58-kDa protein (designated the 56-kDa protein) is the most abundant and is located on the cell surface (Tamura et al. 1985). Other major proteins of 80, 46, 43, 39, 35, 28, and 25 kDa are also located on its surface; three of these proteins, 25-, 28-, and 56-kDa are heat modifiable (Urakami et al. 1985).

The nutritional requirements of the rickettsiae including *Orientia*, as distinct from their host cells, are not known, except that they grow in heavily irradiated cells and in the presence of low levels of cycloheximide (Weiss and Dressler 1958). Under these conditions, rickettsiae incorporate exogenous amino acids and adenine, but not thymidine (Weiss et al. 1972). Optimum metabolic activities require a high concentration of K^+ and physiological levels of Mg^{++}. The activity is stabilized by the presence of protein such as bovine plasma albumin.

The primary substrate is glutamate that is utilized via a glutamate-oxaloacetate transaminase, glutamate dehydrogenase, and the enzymes of tricarboxylic acid cycle (Bovarnick and Snyder 1949). Glutamate metabolism is essential for the maintenance of a high adenylate energy charge (William and Weiss 1978). Glutamine and pyruvate are also utilized, but to a lesser extent (Weiss 1973). Glucose and glucose-6-phosphate are not utilized at all and Rickettsiae including Orientia do not appear to have any of the enzymes commonly associated with glucose metabolism (Coolbaugh et al. 1976).

Orientia is cultured in the yolk sac of chicken embryos, provided the inoculum is relatively large (10^4–10^6 viable cells per egg), and the organism is harvested before the death of the embryos. It is also cultured in cell cultures, mainly on human

endothelial cell line ECV304 and produces small plaques on cell monolayers following 11–17 days of incubation. It grows in the cytoplasm of the host cell, achieving high density in the perinuclear region and acquires a host membrane coat as it emerges from the cell surface (Ewing et al. 1978). It does not penetrate into the nucleus of the host cell. Carbon dioxide enrichment is not necessary for intracellular growth, but otherwise the metabolism of *O. tsutsugamushi* appears to be similar to that of other *Rickettsia* (Kopmans-Gargatiel and Wisseman 1981).

Virulent strains of *O. tsutsugamushi* injected intraperitoneally into mice cause peritonitis, splenomegaly, and death in 10–24 days, but strains vary greatly in virulence. The Karp strain, for example, is more virulent than the Gilliam strain for most out-bread mice. Certain in-bread mice strains are highly resistant to Gilliam strain and their resistance has been shown to be controlled by a single, autosomal dominant gene and not to involve susceptibility to the Karp strain (Groves et al. 1980).

The rickettsiae in general are susceptible to 1 % sodium hypochlorite, 70 % ethanol, 2 % glutaraldehyde, formaldehyde, and quaternary ammonium disinfectants. They are also sensitive to moist heat (121 °C for at least 15 min) and dry heat (160–170 °C for at least 1 h).

4 Pathogenicity

O. tsutsugamushi is transmitted to human host by chiggers. Pathogenesis of scrub typhus may be very complex and involves immune and inflammatory mediators such as cytokines, prostaglandins, leukotrienes, and kinins. As an obligate intracellular organism, *O. tsutsugamushi* must be internalized in host cells. Bacterial invasion is mediated primarily by interaction between bacterial surface components and complementary host receptors, which stimulate host signal transduction pathways required for bacterial entry. *O. tsutsugamushi* exploits host integrin signalling pathways to mediate rearrangements of the actin cytoskeleton for entry into non-phagocytic cells. Engagement of integrin α5β1 molecules on the host cell surface, which is crucial for bacterial entry, plays a role in the early steps of the invasion process by activating protein tyrosine kinase, such as FAK and Src. Subsequent activation of Rho A and local rearrangements of the actin cytoskeleton at the site of infection may promote bacterial uptake. The Type-Specific Antigen 56 (TSA 56), a major outer membrane protein of *O. tsutsugamushi* with fibronectin may mediate engagement of integrin receptors (Cho et al. 2010).

It has been suggested by Koo et al. (2012) that in the host cell, *Orientia* perhaps actively inhibits pyroptosis of infected macrophages, resulting in better survival of the host cells and poorer eradication of the bacterium from infected cells and tissue. Regulation of viability of host cells might play a significant role in efficient infectivity and proliferation of *O. tsutsugamushi*.

O. tsutsugamushi usually infects endothelial cells, macrophages, polymorphonuclear leukocytes (PMNL), and lymphocytes in patient or animal model (Koo et al. 2012). However, in human scrub typhus eschars, it is found to have tropism for dendritic cells

and monocytes rather than endothelium. There is gradual decline in the number of *Orientia* within eschar overtime. Regional lymphadenopathy is common, which coupled with the tropism of *O. tsutsugamushi* to antigen presenting cells (APC) in eschar suggests that one source of replication may be within infected, recirculated APCs in the regional (and other) lymph nodes (Paris et al. 2012).

Orientia grows inside the endothelial cells of heart, lung, brain, kidneys, pancreas, and skin, and within cardiac muscle cells and macrophages located in liver and spleen. *Orientia* has been identified by immune histochemistry using a rabbit polyclonal antibody raised against *O. tsutsugamushi* Karp strain in paraffin embedded archived autopsy tissues of three patients with clinical suspicion of scrub typhus. The location of *Orientia* in endothelium and cardiac myocytes has also been confirmed by electron microscopy (Moron et al. 2001).

5 Clinical Features

Illness varies from mild and self-limiting to fatal. After an incubation period of 6–21 days, onset is characterized by fever, headache, myalgia, cough, and gastrointestinal symptoms. Some patients recover spontaneously after few days. The classic case description includes an eschar where the chigger has fed, regional lymphadenopathy, and a maculopapular rash. Severe cases typically manifest with encephalitis and interstitial pneumonia due to vascular injury.

An eschar at the site of chigger bite can be seen in early disease and is a useful diagnostic clue in scrub typhus though its frequency varies from 7 to 97 % (Paris et al. 2013). Eschars are painless ulcers up to 1 cm in size, with a black necrotic center (resembling the mark of a cigarette burn). Usually, a single eschar is found on the neck, axillae, chest, abdomen, and groin, but multiple eschars have also been documented (Kaushik et al. 2014). Eschar on moist intertriginous surfaces (axilla, scrotum, perianal region) may be missed if not looked into carefully because they may lack the black scab, and appear as shallow yellow based ulcers without surrounding hyperemia (Fig. 18.1. Images 1–3).

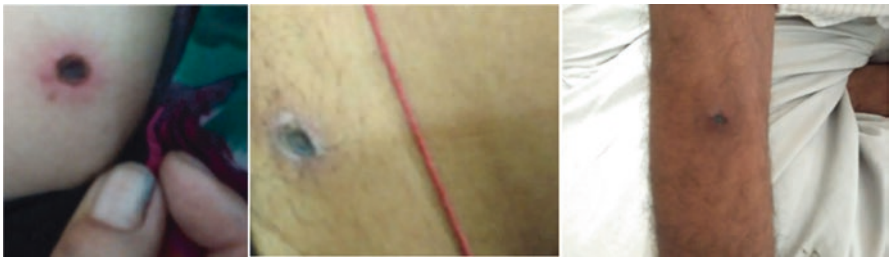

Fig. 18.1 Images 1–3: Eschars in patients with scrub typhus

Though rash is considered as hallmark of rickettsial disease, it is neither seen at presentation nor in all patients. Rash usually becomes apparent after 3–5 days of onset of symptoms. Initially rash is in the form of pink, blanching, discrete maculae which subsequently becomes maculopapular, petechial, or hemorrhagic (Rathi and Rathi 2010). None of these clinical symptoms and signs including eschar are diagnostic of the disease. Therefore, epidemiological factors pertaining to geographical area, habitat, occupation, movement of the subject (vocational or recreational) could assist in reaching a diagnosis of rickettsial disease with certainty and initiating treatment in time.

The complications of scrub typhus usually develop after the first week of illness. Jaundice, renal failure, pneumonitis, acute respiratory distress syndrome (ARDS), septic shock, myocarditis, and meningoencephalitis are various complications known with this disease (Mahajan 2005). Pneumonia is one of the most frequent complications of scrub typhus which manifests as a nonproductive cough and breathlessness and leads to ARDS which could be life-threatening.

Though human disease appears to be of short duration. *O. tsutsugamushi* has been found to persist in mice. The ability to persist in cell culture has also been documented. Antibiotics like chloramphenicol and tetracycline show bacteriostatic effect against *Orientia* in mice. In a study conducted to investigate persistence of viable *O. tsutsugamushi* in six patients who had recovered from scrub typhus, Chung et al. (2012) have shown that on follow up, *O. tsutsugamushi* was isolated from all six patients and nucleotide sequences of isolates serially collected from each patient were identical in all five patients in whom they were studied. In their study, one patient was found to relapse after 2 days of completion of antibiotic therapy, two patients complained of weakness for 1 to 2.5 months after the illness; one patient underwent coronary angioplasty 6 months later; and one patient suffered from a transient ischemic attack 8 months later.

6 Laboratory Diagnosis

Scrub typhus can be diagnosed by direct (isolation and DNA detection) and indirect (serology) methods. For reasons of low sensitivity and expediency, in vitro isolation is rarely used in the clinical setting for the diagnosis of scrub typhus infection, but the ability of culture is imperative for drug susceptibility testing and studies of molecular epidemiology, as well as providing a source for reference cultures for diagnostic tests in an endemic area.

Following samples are to be collected—blood in EDTA for isolation and PCR, blood in plain bulb for serology, and biopsied sample from eschar for PCR. Samples should be transported as soon as possible and any delay should be minimized. However, a study by Luksameetanasan et al. (2007) demonstrated that delay of up to 9 days was shown to be not responsible for lower yield even if samples were transported at ambient temperature. Serum samples should be handled following universal precautions and for isolation of the pathogen the samples should be processed in a Biosafety Level III laboratory.

Weil Felix test: The sharing of the antigens between rickettsia and proteus is the basis of this heterophile antibody test. *O. tsutsugamushi* demonstrates agglutinins to *Proteus mirabilis* strain OXK and not to Proteus vulgaris strains OX2 and OX19. Though this test lacks high sensitivity and specificity, it still serves as a useful and inexpensive diagnostic tool for laboratory diagnosis of scrub typhus and other rickettsial diseases. This test should be carried out only after 5–7 days of onset of fever. Titer of 1:80 is to be considered possible infection. However, baseline titers need to be standardized for each region.

IgM and IgG ELISA: ELISA techniques, particularly immunoglobulin M (IgM) capture assays for serum, are probably the most sensitive tests available for rickettsial diagnosis and the presence of IgM antibodies, indicate comparatively recent infection. In cases of infection with *O. tsutsugamushi,* a significant IgM antibody titer is observed at the end of first week, whereas IgG antibodies appear at the end of second week. Baseline titers need to be established keeping in view the regional variations.

Indirect Immunofluorescence Assay (IFA): This is a reference serological method for diagnosis of scrub typhus and is considered serological "gold standard"; however, cost and requirement of technical expertise limit its wide use.

Indirect Immunoperoxidase Assay (IPA): It gives comparable result as IFA and does not require fluorescent microscope.

Polymerase Chain Reaction (PCR): It is a rapid and specific test for diagnosis. It can be used to detect rickettsial DNA in whole blood and eschar samples. The PCR is targeted at the gene encoding the major 56 kDa and/or 47 kDa surface antigens. The results are best within first week for blood samples because of the presence of rickettsemia *(O.tsutsugamushi, R.rickettsii, R. typhi*, *and R.prowazekii)* in the first 7–10 days.

Drug susceptibility testing: Doxycycline has been used as the drug of choice for scrub typhus. However, reports of doxy-resistant strains in Thailand have aroused interest in new drugs and drug susceptibility testing. Kim et al. (2007b) used flow cytometric technique for the quantification of *Orientia* growth, with and without doxycycline, using specific mononuclear antibodies directed against TSA56. By this method, the authors tested drug susceptibility of the Boryong and AFSC-4 strains of *O. tsutsugamushi.* The use of 2D gel electrophoresis also allows the proteomic comparison of drug-susceptible and -nonsusceptible strains.

7 Treatment

There is paucity of evidence based on randomized controlled trials for the management of rickettsial diseases including scrub typhus (Kim et al. 2007a, Liu and Panpanich 2002). Whenever, rickettsial disease is suspected antibiotic therapy should be started without waiting for laboratory confirmation of the rickettsial infection. Presently, the drugs of choice are doxycycline and azithromycin.

8 Prevention

Scrub typhus is prevalent in an area with about one billion population and one million suffer from the disease (Fig. 18.2). In addition, the difficulty of differentiation of this disease from other causes of fever, reports of refractivity to treatment, and growing tourism in the affected area are the reasons for enhanced concern in developing an effective vaccine (Kelly et al. 2009).

The importance of antigenic variation to the vaccine effort has been described in detail by Kelly et al. (2009). It was underscored early on by Smadel et al. (Smadel et al. 1950, 1951, 1952; Smadel and Elisberg 1965). They showed that homologous strain immunity would persist at least 1–3 years. Although severity of disease was somewhat diminished, immunity to challenge with heterologous *Orientia* strain was short-lived, as short as 1 month. This suggests that a successful vaccine would have to be multipotent. Using mouse model, Bennett et al. (1949) had already shown that inactivated vaccines or antisera prepared against one strain failed to protect against

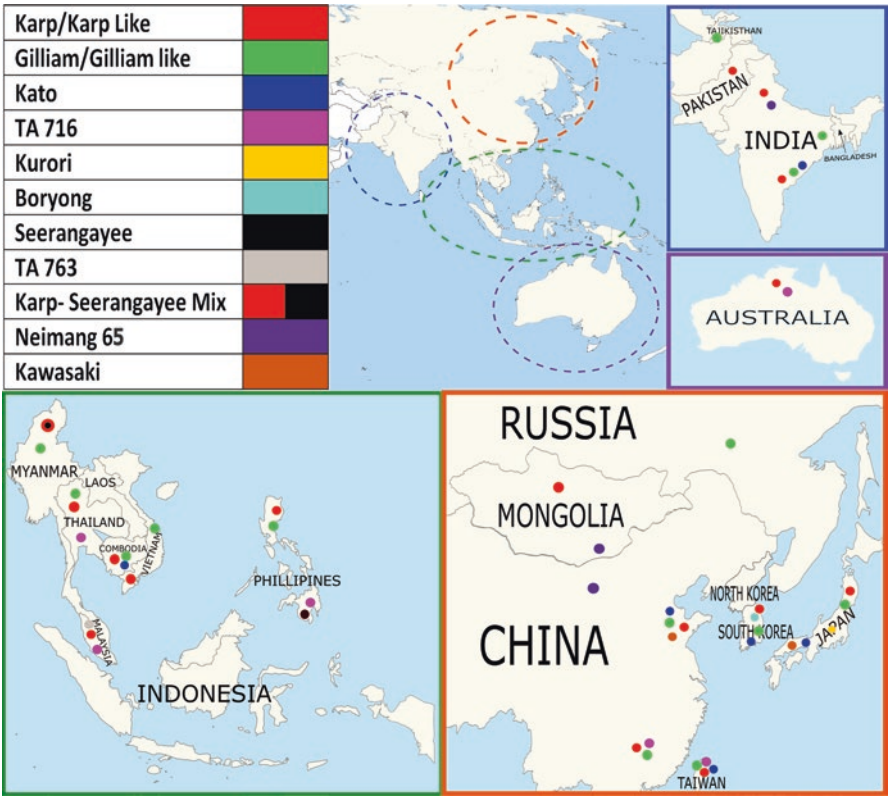

Fig. 18.2 Distribution of major strains of *Orientia tsutsugamushi* (adapted from Kelly et al. 2003 and Varghese et al. 2015)

infection with other strains. Robinson et al. (1981) also demonstrated that silver leaf monkeys showed no resistance to repeat infection with either homologous or heterologous strains at 14 months. Other factors besides short-lived resistance to heterologous repeat infection are also of concern. Whereas a strain with low human virulence might not be considered for incorporation into a vaccine, because of its reduced risk for those infected, a strain with high virulence and thus a greater risk might be considered even if it had a lower prevalence (Kelly et al. 2009).

9 Epidemiological Characterization

Scrub typhus severity and clinical presentation appears to be strain specific, and it varies from extremely mild disease to frequently fatal one in both humans and laboratory animals (Browning et al. 1945; Irons 1946; Irons and Armstrong 1947; Jackson and Smadel 1951). Characterization of *Orientia* strains is important to know variations in disease pattern, its management, and deciding the strains useful for vaccine production. Two methods of characterization have been widely used—serological and molecular. Serological methods such as complement fixation, toxin, and serum neutralization, direct fluorescent antibody assay (DFA), or indirect fluorescent antibody assay (IFA) have been used. Strain identification and strain prevalence for the different regions requires comparison with established "prototypes" available. Early complement fixation characterization compared only Karp, Gilliam, and Kato strains with new isolates (Shishido 1962, 1964), while IFA and DFA analyses starting in late 1970s included selected Thai strains which were shown to be antigenically distinct.

Genetic analysis of the antigen genes of *Orientia* species focused primarily but not exclusively on the 56-kDa cell surface antigen gene. It has resulted in techniques to differentiate genotypes (Stover et al. 1990; Strover et al. 1990; Ohashi et al. 1990). Several new subtypes, such as Japanese Gilliam, Japanese Karp, Kawasaki, Kuroki, Shimokoshi, Neimang-65, etc. in addition to the previously described prototypes Karp, Kato, and Gilliam (Kelly et al. 2009; Nakayama et al. 2010; Yang et al. 2012; Varghese et al. 2015) have been detected (Fig. 18.2). The molecular methods include PCR in conjunction with gel electrophoresis, restriction fragment length polymorphism (RFLP) mapping, and the sequencing of specific PCR products for direct comparison of products from the same gene of multiple *Orientia* strains.

The whole DNA sequence of two strains, the Boryong and the Ikeda have been completed (Chi et al. 2007; Nakayama et al. 2008) and for a number of strains the project is in permanent draft or incomplete state. As per some authors, availability of whole genome sequences for comparison and analysis may lead to increased interest in strain variation and of possible species status of strains within the genus (Chi et al. 2007; Fournier et al. 2003).

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Part VIII
Rickettsia

Chapter 19
Human Rickettsioses: Host Response and Molecular Pathogenesis

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1 Introduction

The genus *Rickettsia* includes Gram-negative bacilli that belong to the class α-proteobacteria. Relatively small AT-rich genomes, fastidious growth requirements, natural transmission through arthropod vectors, and a tropism for endothelial cells in mammalian hosts define these obligately intracellular pathogens (Fig. 19.1) (Blanc et al. 2007; Darby et al. 2007; Fuxelius 2007). Although rickettsiae are traditionally divided into antigenically distinct groups, namely, spotted fever and typhus, recent phylogenetic evidence now categorizes the genus into four main groups: ancestral, typhus, transitional, and spotted fever (Gillespie et al. 2008). The ancestral group consists of *Rickettsia bellii* and *R. canadensis*. The typhus group, responsible for epidemic typhus and endemic typhus, includes only two species: *R. prowazekii* and *R. typhi*. The most diverse group in terms of vectors and disease is the transitional group, comprised of three species: *R. australis*, *R. akari*, and *R. felis*. Nevertheless, the spotted fever group includes the largest species diversification with at least 15 species: *R. aeschlimanii, R. africae, R. conorii, R. heilongjiangensis, R. helvetica, R. honei, R. japonica, R. massiliae, R. montanensis, R. parkeri, R. peacockii, R. rhipicephali, R. rickettsii, R. sibrica,* and *R. slovaca*. There are several other rickettsial species that have yet to be fully characterized, such as *R. asiatica*, *R. hoogstraalii*, and *R. argasii* (Fujita et al. 2006; Lafri et al. 2015).

Rickettsial diseases, such as Rocky Mountain spotted fever (RMSF) and epidemic typhus, have had significant impact on nearly all facets of society and historical events (Table 19.1). RMSF was first described in 1896 by Major Marshall Wood, U.S. Army Medical Corps, in Boise, Idaho, U.S.A. (Thorner et al. 1998). At that time, RMSF was colloquially referred to as “blue disease” or “black measles”

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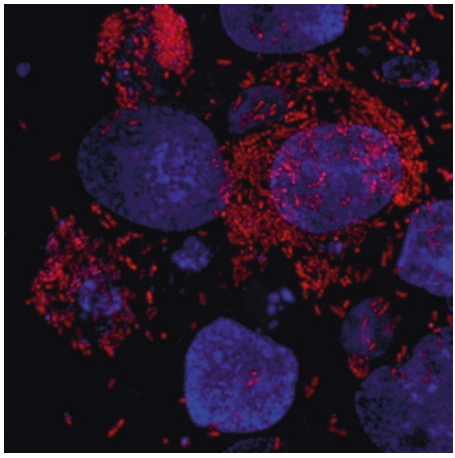

Fig. 19.1 *Rickettsia conorii* in Host Cells. Confocal laser scanning microscopy image of Vero cells infected with *Rickettsia conorii* expressing kusabira orange. DAPI (4′,6′-diamindino-2-phylindole) was used for fluorescent staining (*blue*) of host cell nucleus

(Woodward 1973). Later in 1906, Howard Ricketts demonstrated tick transmission of bacilli and pathology resembling RMSF in guinea pigs. Those ticks associated with transmission include *Dermacentor variabilis* (dog tick), *D. andersoni* (wood tick), *Rhipicephalus sanguineus* (brown dog tick), and *Amblyomma cajennense* (cayenne tick) (Bechah et al. 2008b). The disease, broadly distributed in the United States despite its name, has a geographic range as far north as Canada and as far south as Argentina. The greatest incidence of spotted fever rickettsioses in the United States occurs in a zone that stretches north–south between northern Missouri and southern Arkansas and east–west between mid-Oklahoma and the east coast (Drexler et al. 2016). RMSF and other spotted fever rickettsioses are notifiable diseases in every state of the United States except Hawaii and Alaska (Drexler et al. 2016). If left untreated, RMSF may have a case fatality rate between 20 and 25 % (Smadel 1959; Childs and Paddock 2002). Even in the postantibiotic era, the case fatality rate hovers around 1–2 % (Openshaw et al. 2010). Delayed diagnosis worsens the clinical course of RMSF as it increases the chances of complications and fatal outcome. The key to successful treatment is early administration of appropriate antibiotics (Thorner et al. 1998; Drexler et al. 2016). To date, 2012 has had the highest reported incidence of spotted fever rickettsioses in the United States with approximately 4500 cases (Drexler et al. 2016).

As RMSF is considered to be the most severe spotted fever rickettsiosis, the same holds true for epidemic typhus from the typhus group. Historically, epidemic typhus has been dubbed as the "scourge of the armies," as it was a common infectious disease during wars, famine, and within impoverished areas (Raoult et al. 2006; Zhang et al. 2006; Badiaga and Brouqui 2012). Caused by *R. prowazekii,* epidemic typhus played a significant role in decimating the strength of Napoleon's Grand Army of 1812 (Raoult et al. 2004). During World War II, outbreaks of epidemic typhus were common in military camps, Nazi concentration camps, and battle torn cities. Antibodies for *R. prowazekii* were found in two homeless individuals in Houston, Texas, U.S.A. However, these individuals were not exhibiting

Table 19.1 Disease, in vivo model, and transmission vector associated with *Rickettsia*

Rickettsial group	Species	Disease	Disease model	Vector
Ancestral group				
	R. bellii	Unknown pathogenesis		*Dermacentor varabilis*
	R. canadensis	Unknown pathogenesis		*Haemaphysalis leporis-palustris*
Typhus group				
	R. prowazekii	Epidemic Typhus	BALB/c—IV	*Pediculus humanus corporis; Amblyomma ticks (?);* Flying squirrel ectoparasites
	R. typhi	Endemic Typhus; Murine Typhus	C3H/HeN—IV	*Xenopsylla cheopis; Ctenocephalides felis*
Transitional group				
	R. akari	Rickettsialpox		*Liponyssoides sanguineus*
	R. australis	Queensland tick typhus	BALB/c—IV C57BL/6—IV	*Ixodes holocyclus*
	R. felis	Flea-borne spotted fever		*Ctenocephalides felis; Liposcelis botrychopila*
Spotted fever group				
	R. africae	African tick bite fever		*Amblyomma hebraum; A. variegatum*
	R. conorii	Mediterranean spotted fever; Israeli spotted fever; Astrakhan fever; Indian tick typhus	C3H/HeN—IV	*Rhipicephalus sanguineus; R. pumilio*
	R. heilongjiangensis	Far eastern spotted fever		*Dermacentor silvarum*
	R. helvetica	Unnamed		*Ixodes ricinus*
	R. honei	Flinders Island spotted fever		*Ixodes granulatus*

(continued)

Table 19.1 (continued)

Rickettsial group	Species	Disease	Disease model	Vector
	R. japonica YH	Japanese spotted fever		*Ixodes ovatus*
	R. massiliae	Unnamed		*Rhipicephalus sanguineus; R. turanicus*
	R. monacensis	Unknown pathogenesis		*Ixodes ricinus*
	R. parkerii	American Boutonneuse Fever	C3H/HeJ—IV/ID	*Amblyomma maculatum*
	R. peacockii	Unknown pathogenesis		*Dermacentor andersoni*
	R. raoultii	Scalp eschar and neck lymphadenopathy after tick bite (SENLAT)		*Dermacentor silvarum*
	R. rickettsii	Rocky Mountain spotted fever		*Dermacentor andersoni; D. variabilis; Amblyomma cajennense; Rhipicephalus sanguineus*
	R. sibrica	Sibirian tick typhus; North Asian tick typhus; Lymphangitis-associated rickettsioses		*Dermacentor nuttallii; D. sinicus; Hyalomma asiaticum; H. truncatum*
	R. slovaca	Tick-borne lymphadenopathy (Tibola)		*Dermacentor marginatu; D. reticulates*
	REIS	Unknown pathogenesis		*Ixodes scapularis*

IV intravenous injection; *ID* intradermal injection

signs or symptoms of epidemic typhus at the time of serological testing (Reeves et al. 2008). More recently, outbreaks have occurred in Burundi and Russia (Badiaga and Brouqui 2012). While there is a controversy on whether epidemic typhus originated in the "Americas" or in Europe, today the disease is found worldwide in conjunction with *Pediculus humanus corporis* (human body louse) (Bechah et al. 2008a). The human body louse is the known primary vector of *R. prowazekii*, although there have been reports of its occurrence in *Hyalomma* ticks in Ethiopia, *Amblyomma imitator* ticks from Mexico, and the ectoparasites of *Glaucomys volans volans* (eastern flying squirrel) (Philip et al. 1966; Bozeman et al. 1975; Medina-Sanchez et al. 2005). In the preantibiotic era, the mortality rate for epidemic typhus reached as high as 60 %. However, after the development of antibiotics, the mortality rate dropped to as low as 4 %. Nevertheless, as the disease is prevalent in conditions of famine, war, population displacement, and malnutrition, the mortality rate can still reach as high as 50 % (Bechah et al. 2008a). Interestingly, only *R. prowazekii* has the ability to sustain subclinical latent infection in convalescent persons who can later develop recrudescent typhus, also known as Brill-Zinsser disease. This form of epidemic typhus is generally a milder disease (Bechah et al. 2008a; Sahni et al. 2013). Currently, there are no licensed vaccines for *R. prowazekii* or other rickettsial diseases in the United States or elsewhere.

Association with arthropods is responsible for natural transmission of human rickettsioses through infected vectors. In the case of *Rickettsia* species, these vectors include ticks, cat fleas, rat fleas, mites, and human body lice. The spotted fever group *Rickettsia* and *R. australis* are transmitted through ticks (Fig. 19.2), while the transitional group and typhus group *Rickettsia* are primarily transmitted through nontick vectors. Humans are considered to be "dead-end hosts", as they are not essential to the rickettsial lifecycle, with the notable exception of *R. prowazekii*. In addition, the geographic ranges of rickettsial diseases are limited only by their natural vector's range (Azad and Beard 1998). *Rickettsia*-free ticks acquire spotted fever group *Rickettsia* when the tick takes a sufficient blood meal from a rickettsemic host (Socolovschi et al. 2009). Cofeeding is another situation in which ticks acquire *Rickettsia*. This can occur when an infected tick feeds on a host in close vicinity to *Rickettsia*-free ticks. Although the host may not yet be rickettsemic, the *Rickettsia*-free ticks may acquire the pathogen from the tissue being fed upon by infected tick(s) (Philip 1959). *Rickettsia* have been shown to be passaged by transovarial (to offspring ticks) and transstadial (from one arthropod growth stage to the next) transmission, which ensures their natural maintenance without the need of an intermediate host (Sahni et al. 2013).

The method of transmission is different for *Rickettsia* species transmitted through louse and flea vectors. *Rickettsia prowazekii* (epidemic typhus) is transmitted through *Pediculus humanus corporis* (human body louse; Fig. 19.3), while *R. typhi* (endemic or murine typhus) is transmitted through *Xenopsylla cheopis* (Oriental rat flea). *Rickettsia felis* (cat flea typhus), a transitional group *Rickettsia*, is transmitted by the cat flea, *Ctenocephalides felis*. Another transitional group species, *R. akari*, is unique in that it is the only *Rickettsia* to be transmitted by *Liponyssoides* (house-mouse mite) (Azad and Beard 1998). For these vectors, rickettsiae are not transmitted

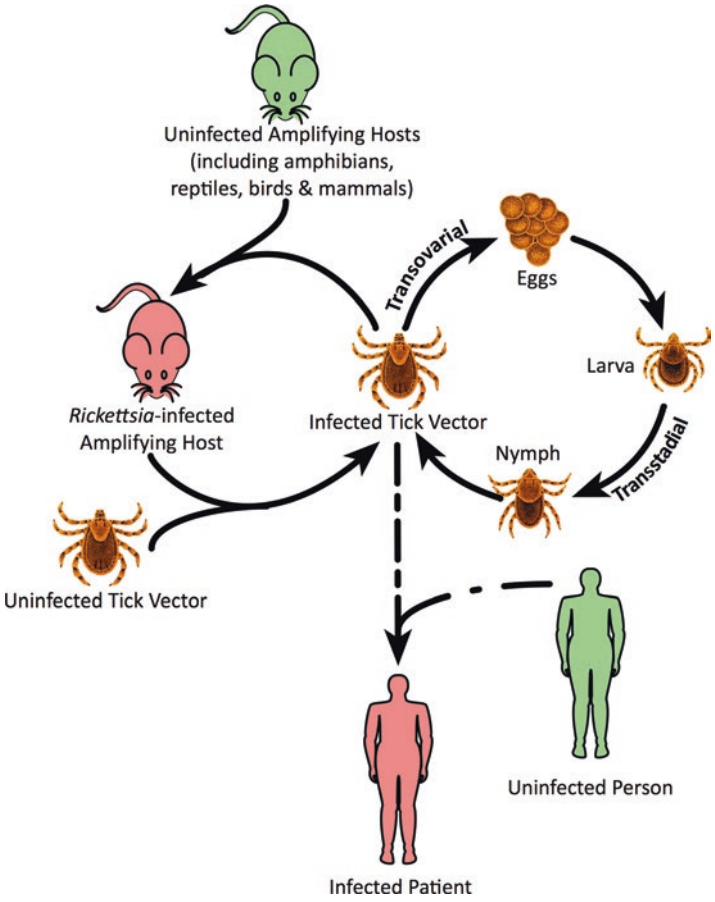

Fig. 19.2 Transmission cycle for the natural maintenance of spotted fever group *Rickettsia*

directly during ingestion of the blood meal from the host unlike tick-borne rickettsiae. Instead, the arthropod vector defecates during the course of taking its blood meal. When the host scratches the bite wound, it accidently introduces *Rickettsia*-contaminated feces into the wound, leading to infection (Azad and Beard 1998). While transovarial passage for flea-borne *Rickettsia* (*R. typhi* and *R. felis*) has been shown, *Rickettsia*-infected lice die within 2 weeks (Farhang-Azad et al. 1985; Azad et al. 1992). Therefore, rapid transmission is essential for the survival of *R. prowazekii*, which lends a possible explanation for recrudescent typhus; i.e., the patients with Brill-Zinsser disease may serve as potential host reservoirs for epidemic typhus.

Historically, ticks, lice, fleas, and mites were considered to be the only vectors for *Rickettsia* species. However, advent of more accurate and sensitive techniques for rickettsial detection in other vectors has enabled expansion of this list to include "nontraditional" vectors (Table 19.1). Mosquito is the generic term that encom-

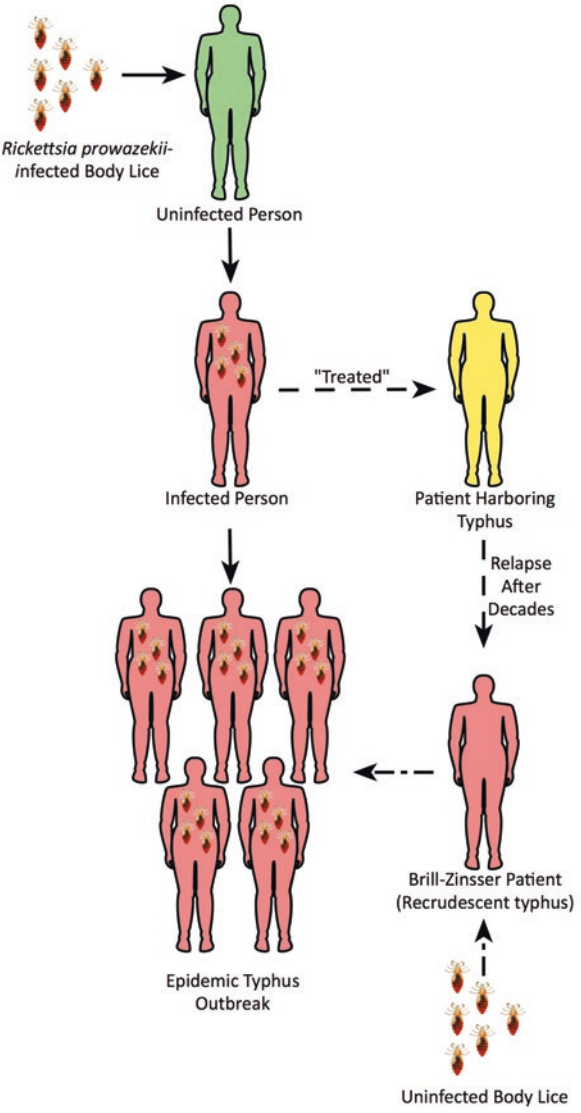

Fig. 19.3 Transmission cycle of *Rickettsia prowazekii*

passes more than 3500 insect species. Using PCR assays, *R. felis* was detected in *Aedes albopictus*, the Asian tiger mosquitoes, from a collection site in Libreville, Gabon (West Coast of Africa) and *Anopheles gambiae* (African malaria mosquito) from Cote d'Ivoire (Socolovschi et al. 2012a, b). Further, *R. felis* was identified from *Liposcelis bostrychophila,* a nonhematophagous arthropod commonly known as the booklouse (Thepparit et al. 2011). Using a variety of molecular tools, 20 additional unclassified rickettsial strains have been identified from a wide range of

vectors that include beetles, flies, wasps, and leeches (Perlman et al. 2006). An unclassified *Rickettsia* sp. that is phylogenetically similar to *R. felis* was isolated from tsetse flies captured in Senegal in western Africa (Mediannikov et al. 2012).

2 Rickettsial Target Cells, Adhesion, and Host Invasion

It is now well accepted that *Rickettsia* preferentially infects vascular endothelial cells during infection of their mammalian hosts. A notable exception to this is *R. akari,* which prefers monocytes and macrophages. As endothelial cells are not professional phagocytic cells, rickettsiae have evolved mechanisms for initiating the invasion process upon adherence to the target cell. Analysis of the rickettsial genome has identified a family of autotransporter genes referred to as *s*urface *c*ell *a*ntigen (*sca*). These are identified as *sca0* through *sca16*. However, evidence suggests that not all 17 Sca proteins are functional and that Sca0 through Sca5 are active (Ngwamidiba et al. 2006; Sears et al. 2012). The Sca proteins are found within the surface layer of the rickettsial membrane and aid in adhesion to the host cell surface (Fig. 19.4 and Table 19.2).

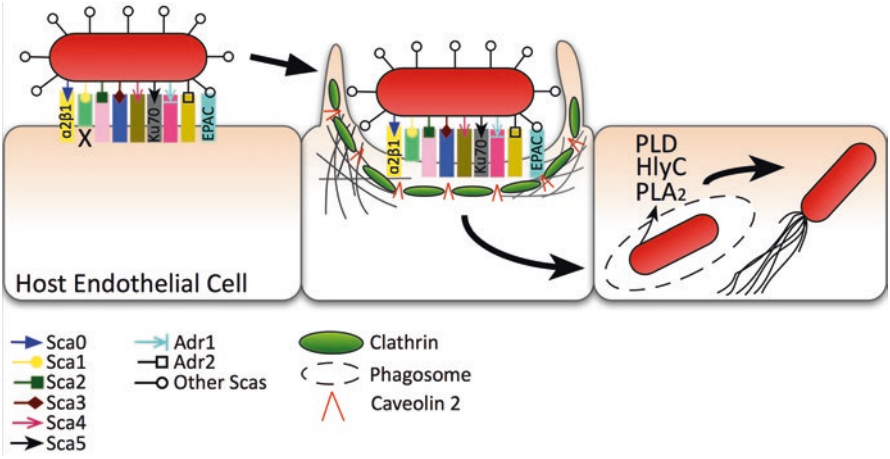

Fig. 19.4 Rickettsial adherence and invasion of host endothelial cells. A schematic depicting major initial stages (adherence, invasion, phagosome escape, and intracellular movement) of rickettsial infection. Activation of host endothelial cells caused by rickettsial adherence induces phagocytosis of the bacteria through immediate-early signaling mechanisms. Activity of phospholipase D (PLD), hemolysin C (HlyC), and patatin-like phospholipase A2 (PLA_2) degrades the phagosome allowing for rickettsial escape into the cytoplasm. Actin-comet tails (spotted fever group) are then formed, which allow for intracellular movement. Black "X" under Sca1 represents that Sca1 aids in adhesion, but not invasion of host endothelial cells

Table 19.2 Rickettsial proteins associated with adhesion to the mammalian host

Rickettsial group	Species	Strain	Sca0	Sca1	Sca2	Sca3	Sca4	Sca5	Adr1	Adr2
Ancestral group										
	R. bellii									
		RML 369-C	–	T	C	P	C	T	C	C
		OSU 85-389	–	T	C	–	C	T	C	C
	R. canadensis	McKiel	C	C	C	P	C	C	C	C
Typhus group										
	R. prowazekii									
		Madrid E	–	P	P	C	T	C	C	C
		Rp22	–	P	P	C	C	C	C	C
		Breinl	–	P	P	C	C	C	C	C
	R. typhi	Wilmington	–	C	C	C	C	C	C	C
Transitional group										
	R. akari	Hartford	P	C	C	P	C	C	C	C
	R. australis									
		Cutlack	C	C	C	P	C	C	C	C
		Phillips	T	P	P	P	T	C	C	C
	R. felis	URRWXCal2	P	C	C	C	C	C	C	C
Spotted fever group										
	Candensis R. amblyommii		C	C	C	P	C	C	C	C
	R. africae	ESF-5	C	C	C	P	C	C	C	C
	R. conorii	Malish 7	C	C	C	P	C	C	C	C
	R. heilongjiangensis	54	C	C	C	P	C	C	C	C
	R. honei	RB	T	C	C	P	C	C	C	C

(continued)

Table 19.2 (continued)

Rickettsial group	Species	Strain	Sca0	Sca1	Sca2	Sca3	Sca4	Sca5	Adr1	Adr2
	R. japonica	YH	C	C	C	P	C	C	C	C
	R. massiliae									
		MTU5	C	C	C	P	C	C	C	C
		AZT80	C	C	C	P	C	C	C	C
	R. montanensis	OSU 85	C	C	C	–	C	C	C	C
	R. parkerii	Portsmouth	C	C	C	P	C	C	C	C
	R. peacockii	Rustic	P	–	C	–	C	C	C	C
	R. philipii	364D	C	C	C	P	C	C	C	C
	R. rhipicephali									
		3-7-female6-CWPP	C	C	C	–	C	C	C	C
	R. rickettsii									
		Sheila Smith	C	C	C	P	C	C	C	C
		Iowa	T	C	C	P	C	C	C	C
		R	C	C	C	P	C	C	C	C
		Brazil	C	C	C	P	C	C	C	C
	R. sibrica									
		mongolitimonae HA-91	T	C	C	P	C	C	C	C
		sibirica BJ-90	T	C	C	P	C	C	C	C
	R. slovaca									
		13-B	C	C	C	P	C	C	C	C
		D-CWPP	C	C	C	P	C	C	C	C
	REIS		C	C	P	–	T	C	C	C

C = Expression protein, T = Truncated protein, P = pseudogene, – Not present

Sca0, more commonly known as *rompA* for *r*ickettsial *o*uter *m*embrane *p*rotein A, is encoded in the majority of spotted fever group *Rickettsia*, but the functional gene is absent in the typhus group (*R. prowazekii* and *R. typhi*) (Blanc et al. 2005; Sears et al. 2012), although *R. prowazekii* does encode a 369-bp fragment of *ompA* (Uchiyama 2012). The size of the corresponding protein varies with the rickettsial species. For example, the molecular weight of *R. rickettsii* OmpA is approximately 247 kDa, while that of *R. conorii* is approximately 224 kDa (Chan et al. 2010). Despite a 96.6 % genome similarity to virulent *R. rickettsii* strain Sheila Smith, avirulent *R. rickettsii* strain Iowa truncates *ompA* 660 bp beyond the start codon by virtue of an early termination codon. Although there are other notable differences such as single nucleotide polymorphisms and insertion/deletions (indels) between these strains, premature termination of OmpA is an important mechanism for the avirulent phenotype of strain Iowa (Ellison et al. 2008; Noriea et al. 2015). Evidence suggests that OmpA is processed further and migrates as a 190 kDa protein on an SDS-PAGE gel (Vishwanath 1991). The variation in size between different species and strains is attributed to the number of tandem repeat sequences. Currently, the function of these repeat sequences is unknown, but their presence hints a conserved function. Evidence further suggests that *sca0* encodes for an adhesion protein. With monoclonal antibodies directed against OmpA, *R. rickettsii* adhesion to host target cells can be inhibited as much as 90 %. In addition, a competition assay with purified OmpA and *R. rickettsii* showed inhibition of rickettsial adhesion (Li and Walker 1998). Expression of rickettsial OmpA promotes *Escherichia coli* adherence to HeLa cells and human microvascular lung endothelial cells (HMVEC-L) (Hillman et al. 2013). Further, not only is OmpA sufficient to promote adherence, but the protein also mediates invasion of *E. coli* into host cells as shown by a gentamicin protection assay. A competitive inhibition assay lends further support to this observation as purified OmpA inhibits *R. conorii* invasion of host cells (Hillman et al. 2013). Rickettsial OmpA interacts with $\alpha_2\beta_1$ integrin on the host cell membrane to promote internalization through an unknown mechanism. Genetic manipulation of *Rickettsia* is extremely limited due to its obligately intracellular lifestyle and reductive evolution. However, recent application of the group II intron-based system (TargeTron vector technology, Sigma) to successfully introduce a premature stop codon in the OmpA of *R. rickettsii* strain Sheila Smith (virulent) reveals no significant effects on pathogenicity in a guinea pig model of infection and an unaltered phenotype in cell culture (Noriea et al. 2015).

While *sca0* is only found in spotted fever group *Rickettsia*, *sca5,* more commonly known as *rompB,* is present in all *Rickettsia* except *R. canadensis* (Ngwamidiba et al. 2006). As a surface cell antigen, it is the most predominant protein found in the proteinaceous surface layer of rickettsial cell wall (Chan et al. 2010). The protein shows a high degree of conservation (70–95 %) among rickettsial species, which suggests a universal function within the genus. The OmpB protein is composed of three domains: (1) signal peptide, (2) passenger domain, and (3) β-barrel transmembrane domain. Similar to other Sca proteins, the 168 kDa OmpB preprotein is cleaved to form a processed 120 kDa passenger domain and a 32 kDa β-barrel domain. The passenger domain remains attached to the outer leaflet of the

outer membrane (Hackstadt et al. 1992), whereas the signal peptide in conjunction with the Sec translocon translocates the protein across the inner membrane and deposits it in the periplasmic space. Following this event, the β-barrel transmembrane domain implants into the bacterial outer membrane to form a pore, the mechanism for which is not yet fully appreciated. The passenger domain is then fed through the β-barrel pore and subsequently cleaved (Chan et al. 2010). Evidence suggests that OmpB is also sufficient for both adhesion and invasion into host cells (Chan et al. 2009). OmpB antibodies prevent the internalization of *R. japonica*, a spotted fever group *Rickettsia*, into Vero cells. *E. coli* transformed with *ompB*-containing plasmids quickly adhere to target host cells (by 20 min) and become internalized by about 120 min postinfection (Uchiyama et al. 2006). OmpB has been shown to interact with Ku70 in *R. conorii*. Interestingly, Ku70 serves in key nuclear processes such as DNA repair, chromosome maintenance, and V(D)J recombination. Although primarily a nuclear protein, it can be found in the cytoplasm and plasma membrane of mammalian cells (Koike 2002). In the cytoplasm, Ku70 inhibits Bax-mediated apoptosis, but it functions as an adhesion molecule in the membrane and triggers signal transduction within the lipid rafts (Koike 2002; Lucero et al. 2003; Monferran et al. 2004; Martinez et al. 2005).

Although surface cell antigens OmpA and OmpB have been the major focus of investigations for rickettsia adhesion and invasion, attention to *sca* genes has also been gaining momentum. *Sca1* genes have been found in all rickettsial species with two caveats. For *R. prowazekii,* the *sca1* gene is split into three consecutive ORFs as opposed to a single gene found in other rickettsial species. *Rickettsia canadensis* also carries a split *sca1* gene. RT-PCR and western blotting demonstrate *sca1* expression in *R. conorii* and *R. typhi* (Ngwamidiba et al. 2006; Riley et al. 2010; Sears et al. 2012). Surface localization using immunofluorescence microscopy provides further evidence that Sca1 is expressed on the bacterial membrane (Riley et al. 2010). This is to be expected as the protein consists of a β-barrel transmembrane domain similar to OmpA and OmpB (Blanc et al. 2005). Interestingly, SDS-PAGE analysis enabled identification of a protein with the molecular mass of approximately 130 kDa that resembled peptides found in OmpA, OmpB, and Sca1. Using *R. conorii* lysates, this protein was confirmed using an anti-Sca1 and hypothesized to be a processed fragment from the full-length Sca1 protein (Riley et al. 2010). When the full-length *R. conorii sca1* was transformed into *E. coli* as a heterologous system, the expressed protein was sufficient for *E. coli* adherence to cultured mammalian cells. However, unlike OmpA and OmpB, *R. conorii* Sca1 was not sufficient to induce invasion into host cells (Riley et al. 2010). The mammalian receptor that Sca1 interacts to facilitate adhesion is currently unknown.

Found primarily in spotted fever *Rickettsia*, *sca2* is fragmented in *R. prowazekii* (Blanc et al. 2005; Ngwamidiba et al. 2006; Sears et al. 2012). Originally, *R. typhi* was also reported to possess a fragmented *sca2* gene, but recent data suggest that *R. typhi* does indeed encode a complete *sca2* (Dreher-Lesnick et al. 2008; Sears et al. 2012). Within the spotted fever group, the Sca2 is strongly conserved between species as the similarity ranges from 90 to 95 % and identity between 89 and 94 % (Cardwell and Martinez 2009). Sca2 consists of a short N-terminal signal sequence,

a passenger domain, and an autotransporter domain. Although predicted to be approximately 200 kDa, western immunoblotting using anti-Sca2 demonstrated a 150 kDa protein in *R. conorii*. This indicated that Sca2 was processed in a manner similar to other Sca proteins (Cardwell and Martinez 2009). However, in analyzing the *sca2* gene in *R. typhi*, it was found that the passenger domain differed considerably from that of spotted fever *Rickettsia* in sequence length, amino acid identity, and the number of repeat regions (Sears et al. 2012). Consistent with the *sca2* from *R. parkerii,* the *sca2* for *R. typhi*, *R. prowazekii,* and *R. bellii* contains five WH2 domains, which differ in their location in comparison to other spotted fever *Rickettsia*. When compared to *R. akari*, which for an unknown reason encodes two *sca2* genes of varying lengths (1498 bp and 1531 bp), the WH2 domains also differ. It has been demonstrated that *R. typhi* Sca2 is transcribed and translated during infection of L929 cells (Sears et al. 2012). Using *E. coli* as a heterologous expression system, *R. conorii* Sca2 was shown to be sufficient to promote adherence to a variety of mammalian cells, including HeLa, Vero, Ea.hy926, and human lung microvascular (HLMV) cells (Cardwell and Martinez 2009). Gentamicin protection assays further confirmed that Sca2 was sufficient for invasion of *E. coli* into mammalian epithelial and endothelial cells. However, like Sca1, the mammalian receptor for Sca2 also remains currently unknown. Interestingly, Sca2 is unique in that it may mimic eukaryotic formins since it nucleates unbranched actin filaments (Madasu et al. 2013). Immunofluorescence microscopy clearly indicates that *R. parkeri* Sca2 localizes with the polar actin tails (Haglund et al. 2010). Furthermore, knockout of Sca2 in *R. rickettsii* results in disruption of actin tail assembly and motility (Kleba et al. 2010). The function and pathways associated with Sca2 are currently being studied.

Little is known about the remaining *sca* genes and proteins. Interestingly, evidence derived from bioinformatics suggests that *sca3* is only present in *R. prowazekii, R. typhi,* and *R. felis*. The spotted fever and ancestral group *Rickettsia* only display pseudogenes for *sca3* (Sears et al. 2012). On the other hand, *sca4* is present in all rickettsial groups. However, the *sca4* gene is split in the avirulent *R. prowazekii* strain Madrid E but is complete in the virulent *R. prowazekii* strain Rp22 (Sears et al. 2012). While present in *R. bellii,* an ancestral group *Rickettsia*, *sca4* appears to be absent in *R. canadensis* (Ngwamidiba et al. 2006). Quantitative RT-PCR data suggest that *sca3* and *sca4* are active in L929 cells infected with *R. typhi*. This is further supported by in vivo immunofluorescence assays with anti-Sca3 and anti-Sca4 in cat fleas (Sears et al. 2012). Other than transcription and surface expression, not much is known about their function in adherence or invasion (Gillespie et al. 2015). Based on the evidence that *Shigella* encodes an activator of vinculin to bind to the actin cytoskeleton, a search of vinculin-binding sites revealed that *sca4* encodes two such sites in its C-terminal half (Park et al. 2011). Vinculin is an inactive helix bundle protein activated by interrupting the head–tail interaction. Gel-shift mobility assays demonstrated that Sca4 bound to and activated vinculin through both of the vinculin-binding sites. Fluorescence microscopy also showed colocalization of Sca4, vinculin, and F-actin, suggesting that Sca4 activates vinculin and binds to actin cytoskeleton (Park et al. 2011). How Sca4, known to lack an

autotransporter domain, is transported to the rickettsial surface remains unclear at this point (Blanc et al. 2005; Gillespie et al. 2015).

Rickettsial adhesion is not limited to the surface cell antigens (sca) family. A proteomics study using overlay assays identified two proteins of 30 and 32 kDa, respectively, in *R. conorii* and *R. prowazekii*. Mass spectroscopy identified the 32 kDa protein as OmpB and the 30 kDa as an unknown protein, which was encoded by RC1281 in *R. conorii* and RP828 in *R. prowazekii* (Renesto et al. 2006). This protein was later named *ad*hesion of *r*ickettsiae (Adr) 1 (Vellaiswamy et al. 2011b; Gillespie et al. 2015). A BLAST search of both genomes revealed an additional gene, RC1282 and RP827, with similarity to RC1281 and RP828, respectively (Renesto et al. 2006). These were later named Adr2 (Vellaiswamy et al. 2011b). Expression of Adr1 (RC1281) on *R. conorii* and Adr1 (RP828) and Adr2 (RP827) on *R. prowazekii* surface was demonstrable using an overlay assay. The presence of Adr2 (RC1282) on the surface of *R. conorii* has yet to be confirmed (Renesto et al. 2006; Vellaiswamy et al. 2011b). Further, Adr2 was shown to be sufficient for adhesion and entry into L929 cells and nearly 50 % of *R. prowazekii* were prohibited from gaining entry into the cells in the presence of antibody against Adr2 (Vellaiswamy et al. 2011b).

Rickettsia are intracellular bacteria living freely within the nutrient-rich host cytosol. This means that the bacteria must adhere to the target cell, be engulfed by the host cell through a phagocytic vacuole or similar structure, and finally degrade the phagosome to free themselves in the cytosol (Teysseire et al. 1995). In this regard, rickettsial internationalization into a host cell is rather quick and efficient. Transmission electron microscopy showed that as early as 3 min, nearly 60 % of *Rickettsia* had already adhered to the cell membrane, of which 40 % were already internalized. At 6 min postinfection, 60 % of the bacteria were internalized into the host cell, while at 12 min, 90 % were internalized. Frequently, the observation of phagosome lysis occurred at this stage (Teysseire et al. 1995). As phagosome escape is just as important as adherence and invasion of host cells, *Rickettsia* complete this stage rapidly and efficiently as well. After 30 min, nearly 35 % of internalized *Rickettsia* escape the phagosome in Vero cells. At 50 min postinfection, this number nearly doubles to 69 % (Whitworth et al. 2005). This entry into host cells occurs through a zipper-like action initiated through a receptor-mediated invasion (Chan et al. 2010). Host actin is polymerized through the activation of a cascade involving phosphoinositide 3-kinase, c-Src, protein tyrosine kinases, and Cdc42 resulting in the activation of Arp2/3, an actin nucleating complex (Martinez and Cossart 2004). Actin rearrangement is reported to occur through a WASP homolog (Campellone et al. 2008). OmpB internalization is dependent on c-Cbl, clathrin, and caveolin-2-dependent endocytosis (Chan et al. 2009). Once the bacterium has been phagocytized, it must escape the phagosome. Currently, there is evidence that *Rickettsia* use three proteins to accomplish escape into host cytoplasm. Hemolysin C (TlyC) has been implicated in such a role due to its hemolytic activity when expressed in a heterologous system (Radulovic et al. 1999). Phospholipase D (Pld), although not annotated as such in the *R. prowazekii* genome, was later characterized in the genomes of *R. conorii* and *R. prowazekii*. This was somewhat surprising consider-

ing the virulence activity of this gene in other intracellular bacteria (Renesto et al. 2003). It was not until these genes were expressed in the *Salmonella* heterologous system, however, that they could be strongly correlated to the phenomenon of phagosome escape. It was found that *tlyC* and *pld* were both transcribed early during the phagosomal escape, but *tlyA* and *pat1* were not. When *tlyC* and *pld* were expressed in *Salmonella,* either of these genes was sufficient to allow *Salmonella* to escape the phagosome (Whitworth et al. 2005). Within *R. typhi*, two genes, RT0590 and RT0522, encode for patatin-like phospholipase A_2 (PLA_2) proteins. Although long hypothesized to aid in phagosomal escape, it was not until recently that this was demonstrated experimentally. Using immunofluorescence assays, *R. typhi* pretreated with anti-Pat1 or anti-Pat2 exhibited reduced phagosome escape in Vero76 cells (Rahman et al. 2013). Once *Rickettsia* escape from the phagosome, the bacteria grow and replicate freely in the cytoplasm of the host cell.

3 Endothelial Interactions and Inflammatory Response

During infection of their mammalian hosts, *Rickettsia* are able to spread to the lungs, brain, liver, and other organs (Fig. 19.5) through the vascular system or to the regional lymph nodes through the lymphatic endothelium (Walker and Gear 1985). Lining the interior of small- and medium-sized blood vessels as a selective, permeable barrier between the blood and extravascular tissue, endothelial cells perform critical physiological functions and maintain hemostasis (Sahni et al. 2013). It is also important to consider that endothelial cells in different organ systems are functionally and phenotypically heterogeneous in their receptor expression profiles, permeability properties, and activation. When injured by bacterial infections such as rickettsioses, endothelial cells promote leukocyte interactions, secrete pro-inflammatory cytokines and chemokines, express adhesion molecules, and experience dysfunction or damage leading to increased vascular permeability (Fig. 19.6) (Rydkina et al. 2006; Walker and Ismail 2008). Although not considered professional phagocytic cells,

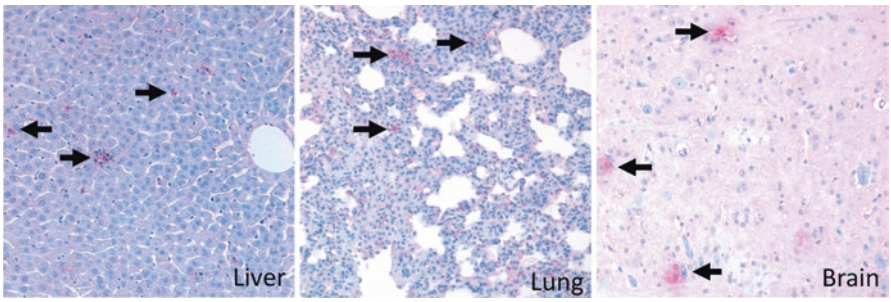

Fig. 19.5 *Rickettsia* dissemination in tissues. Immunohistochemical staining of *Rickettsia typhi* in the liver, lung, and brain of C3H/HeN mice at day 7 postinfection. Abundant rickettsial antigen (*black arrows*) can be seen in all tissues, demonstrating systemic dissemination of rickettsiae

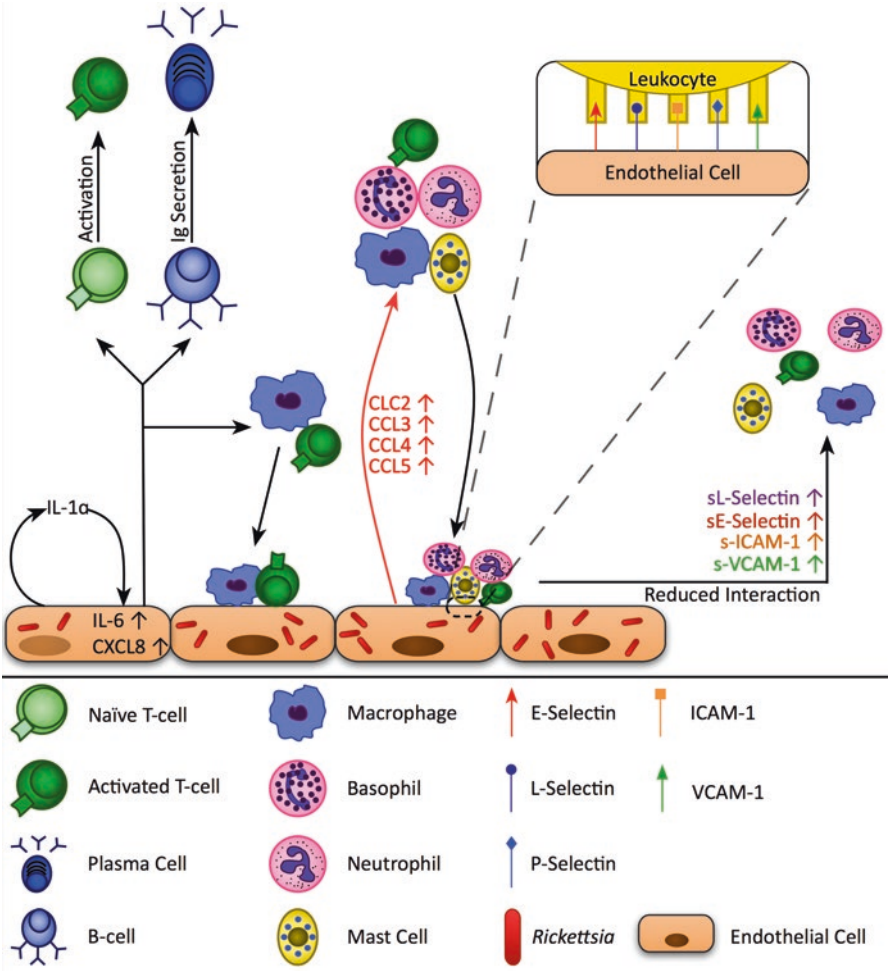

Fig. 19.6 Endothelial cell activation and inflammatory response to rickettsial infection. A summary of the current state of knowledge regarding endothelial cell activation postrickettsial infection and subsequent inflammatory response. Activation of host endothelium leads to activation of lymphocytes and recruitment of leukocytes through the secretion of pro-inflammatory cytokines

they are capable of internalizing bacteria and providing a broad range of immune responses via endothelial activation, antigen presentation, and recruitment of leukocytes to the sites of infection. Using in vitro and in vivo models of rickettsioses, altered levels of antioxidant enzymes, accumulation of intracellular reactive oxygen species (ROS), and reduced level of protective thiols have been documented to lead to oxidative stress-mediated endothelial cell injury (Mansueto et al. 2012). As a host defense against infection, tumor necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ) induce superoxide anion (O_2^-) and hydroxyl radical (OH^-) generation through autocrine and paracrine signaling pathways (Ismail et al. 2002).

Further, endothelial cells are capable of altering their gene transcription profile thereby mediating inflammation through the secretion of cytokines and chemokines. These include interleukins (IL) 1, 3, 7, 14, and 15; tumor necrosis factor-α (TNF-α); and colony stimulating factors (CSF) 1, 2, and 3. Conversely, endothelial cells also express anti-inflammatory cytokines, such as IL-5, 6, 8, and 11 and transforming growth factor-β (TGF-β). In experimental models of rickettsial infection, cell surface expression and/or secretion of platelet derived growth factor (PDGF), vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and E-selectin have been reported. Also secreted are chemokine ligands CCL2, CXCL1, CXCL5, CXCL9, and CXCL10 (Mantovani et al. 1998; Valbuena et al. 2002). In order to promote adhesion and capture of circulating leukocytes during the acute phase of a rickettsial infection, expression of E-, P-, and L-selectin molecules on the surface of endothelial cells enables the interaction with Sialyl Lewis^X counter-receptor on the surface of leukocytes (Crockett-Torabi and Fantone 1995; Mansueto et al. 2012). Within two weeks of symptom onset, proteolytic cleavage of their membrane-bound counterparts on activated leukocytes occurs and soluble forms of sL-selectin, sE-selectin, s-ICAM-1, and s-VCAM-1 are released into the plasma. Alternatively, transcript variants may also produce soluble forms of these adhesion molecules that are shed into the plasma. The presence of these shed particles in the plasma might reduce endothelial cell and leukocyte interactions, thus substantially reducing leukocyte infiltration (Vitale et al. 1999; Mansueto et al. 2012). Most likely, this increases anti-inflammatory cytokine response at a later phase of acute infection in order to reduce the deleterious effects of pro-inflammatory cytokines.

During rickettsioses, NF-κB and MAPK, two major signaling cascades, are activated in target endothelial cells. As a major transcription factor, *R. rickettsii* is able to activate nuclear factor-κB (NF-κB) in a "cell-free" system through an as yet unidentified protease activity (Sahni et al. 2003; Clifton et al. 2005). NF-κB regulates CCL2 and CXCL8, the chemokines released in response to infection. When IκB, an inhibitor of NF-κB, is phosphorylated by IκB kinase (IKK), NF-κB is released from the NF-κB–IκB complex and translocates to the nucleus, thereby increasing the magnitude of the inflammatory response (Clifton et al. 2005). In addition to NF-kB activation, rickettsial infections also activate mitogen-activated protein kinases (MAPK) signaling cascades. Extracellular signal-regulated kinases (ERKs), p38 MAPK, and c-Jun NH2-terminal kinase (JNK) are three major modules of the MAPK pathway, but these can be activated independently or constitutively. During *R. rickettsii* and *R. conorii* infection, p38 MAPK pathway is selectively activated in endothelial cells. Rickettsial adherence to endothelial cells induces the phosphorylation of p38, enhancing bacterial invasion into the host cells. The inhibition of p38 signaling pathway does not affect the NF-κB activation response but limits the expression of CCL2 and CXCL8 (Rydkina et al. 2005, 2007).

Chemokines are expressed at relatively low basal levels in endothelial cells and are well known for their roles as chemoattractants, in T-cell maturation, and in homeostatic functions. In response to *R. conorii* infection, human umbilical vein endothelial cells (HUVECs) express IL-6 and CXCL8 via an IL-1α-dependent pathway. Autocrine signaling of IL-1α through IL-1 receptor (IL-1R) and binding to the

endothelial cell surface induces the secretion of IL-6 and CXCL8. The secreted IL-6 and CXCL8 are likely involved in T-cell differentiation and leukocyte infiltration into the vasculature, respectively (Kaplanski et al. 1995). Macrophage and monocyte interactions with endothelial cells are attributed to increased expression of CCL2, CCL3, CCL4, and CCL5. CXCL8 and CCL8 are responsible for the recruitment of monocytes, lymphocytes, and other polymorphonuclear leukocytes, while CXCL9 and CXCL10 are T-cell chemoattractants. In summary, induced expression and secretion of cytokines and chemokines by infected endothelial cells occurs predominantly through NF-*k*B and p38 MAPK pathways (Kaplanski et al. 1995; Valbuena et al. 2003; Valbuena and Walker 2004; Rydkina et al. 2005).

3.1 Pattern Recognition Receptors

Host defense against rickettsial infection is through the innate and acquired immune systems. Although endothelial cells represent the primary target, spotted fever group *Rickettsia* have been shown to invade certain immune regulatory cells, such as macrophages and peripheral blood monocytes, parenchymal cells such as hepatocytes, and perivascular smooth muscle cells as secondary targets (Feng and Walker 2000). The host's first line of defense is the innate immune system, which must recognize "self" from "nonself." The immune system accomplishes this by the recognition of pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS) and peptidoglycan, through pattern recognition receptors (PRRs). The membrane-bound Toll-like receptors (TLRs) and the cytoplasmic NOD-like receptors (NLRs) are two such PRRs.

Evolutionarily conserved TLRs are responsible for pathogen recognition, signal transduction, and tailoring the innate and adaptive immune responses (Takeda and Akira 2005). Currently, there are ten known TLR members (TLR1 to TLR10) expressed in humans and twelve (TLR1 to TLR9, TLR11 to TLR13) are reported in mice (Takeda and Akira 2015). A TLR is composed of two components, an extracellular domain and a cytoplasmic Toll-IL-1 receptor (TIR) domain. The extracellular domain contains a variable number of leucine-rich repeats (LRRs), which are responsible for PAMP recognition. Exactly how this recognition occurs through LRRs remains unclear. The cytoplasmic TIR domain is highly similar to the cytoplasmic IL-1 receptor domain, but the extracellular domain is unrelated to the IL-1 receptor (Takeda and Akira 2015). The activation of specific TLRs either recruits one or both adaptor molecules, namely, myeloid differentiation primary response gene (MyD)88 and TIR domain containing adaptor protein (TIRAP), for downstream signaling. TLR3 and TLR4 further constitute two unique scenarios upon activation. In addition to MyD88 and TIRAP, TLR4 can also recruit two additional adaptor molecules, TIR-domain-containing adapter-inducing interferon-β (TRIF) and TRIF-related adaptor molecule (TRAM), resulting in the activation of either MyD88-dependent or MyD88-independent pathways. On the other hand, TLR3 is restricted to following only a TRIF-mediated signaling pathway (Takeda and Akira 2005).

TLRs trigger downstream signaling responses by recognizing specific PAMPs. TLR2 is unique in that it recognizes a variety of PAMPs, such as peptidoglycan and lipopeptides, as it can either homodimerize or heterodimerize with TLR1 and TLR6. TLR2-deficient HEK293 cells cotransfected with the receptor complex TLR2, CD14, and MD2 and stimulated with *R. akari* activate NF-κB leading to the expression of TNF-α and CXCL8 (Quevedo-Diaz et al. 2010). LPS in the cell wall of *Rickettsia* and other Gram-negative bacteria activates TLR4-mediated signaling for clearance of intracellular bacteria (Jordan et al. 2009). Evidence from TLR4-deficient C3H/HeJ mice succumbing to a sublethal infecton with *R. conorii* demonstrates the importance of TLR4 signaling in rickettsial clearance (Jordan et al. 2008). Moreover, dendritic cells (DCs) recognize and induce a protective immune response against *R. conorii* and promote clonal expansion of natural killer (NK) cells (Jordan et al. 2009). TLR9 recognizes CpG oligodeoxynucleotides (ODN) found within bacterial DNA. CpG ODN administration into indoleamine 2,3-dioxygenase (IDO)-deficient C57BL/6J mice protects against a lethal dose of *R. australis,* suggesting a protective role for TLR9 during rickettsial infection (Xin et al. 2012).

NLRs are a class of evolutionary conserved cytoplasmic receptors that can sense intracellular bacteria and bacterial cell wall components. In particular, nucleotide-binding oligomerization domain (NOD) 1 and NOD2, members of the NLR subfamily C, have been implicated in recognition of PAMPs associated with intracellular bacteria. Both NOD1 and NOD2 can trigger cytokine secretion via NF-κB and p38MAPK pathways (Strober et al. 2006; Carneiro et al. 2008). Evidence from recent studies demonstrates that both NOD1 and NOD2 can serve as the sensor of a divergent array of peptidoglycans. However, NOD1 and NOD2 pathways are not yet explored in rickettsial infections. Further investigations of the potential cross-talk between TLR2 receptor complex and NOD1 and NOD2 signaling pathways should reveal exciting new understanding of antirickettsial immune response mechanisms.

3.2 Host Innate Immune Response

NK cells are innate immune cells that confer responsiveness to rickettsiae through secretion of IFN-γ, perforin, and granzyme (Fig. 19.7) (Fang et al. 2012). The major function of IFN-γ originating from NK cells is to initiate the defense against and clearance of the invading organisms. Using C3H/HeN mouse models, NK cells are activated through TLR4 stimulation and recruited to draining lymph nodes by TLR-induced DCs. IFN-γ produced by these cells assists in the T-helper (Th)1 cell response and activation of macrophages (Jordan et al. 2009). Rickettsial growth is restricted by activated macrophages due to the production of nitric oxide (NO) (Feng and Walker 2000). Further, recognition of *Rickettsia* by antigen-presenting cells (APC), such as DCs, induces an inflammatory response, which ultimately dictates the development of adaptive immunity (Banchereau et al. 2000; Bechelli et al. 2016).

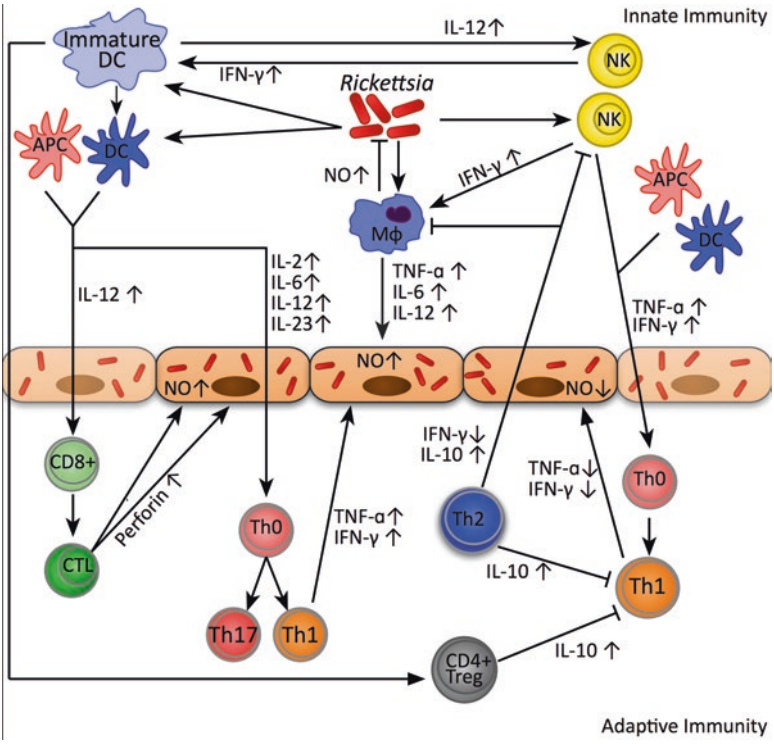

Fig. 19.7 Innate and adaptive immune response to Rickettsial infection. A schematic depicting the host's innate (*top*) and adaptive (*bottom*) response to rickettsioses. The response is delicately balanced through the secretion of both pro-inflammatory and anti-inflammatory cytokines

DC secretion of pro-inflammatory cytokines IL-2, IL-6, IL-12, and IL-23 also facilitates clonal production of Th1 and Th17-mediated adaptive immune response to rickettsial pathogens (Jordan et al. 2007, 2008).

Rickettsia can invade and survive within monocytes and macrophages (Feng and Walker 2000). Along with infected endothelial cells, *R. conorii*-infected macrophages are evident in the experimental C3H/HeN mouse model (Walker et al. 1994). Once inside macrophages, rickettsial death is caused by the starvation of tryptophan and activity of indoleamine 2,3-dioxygenase (MacKenzie et al. 2007). *Rickettsia akari* is unique among rickettsiae as the only species that preferentially infects macrophages and monocytes (Azad and Beard 1998). Mouse peritoneal macrophages infected with *R. akari* demonstrate no remarkable production of TNF-α or TGF-β, but a strong expression of IL-1β and IL-6 is evident. Moreover, *R. typhi* infects peritoneal macrophages of C3H/HeN-CrlBr mice, resulting in increased production of pro-inflammatory cytokines IL-1β and IL-6 as well as TNF-α and TGF-β. Despite a number of similarities between these two species, the differential expression of cytokines may potentially explain the biological differences between *R. akari* and *R. typhi* (Radulovic et al. 2002).

APCs and NK cells activated by *Rickettsia* produce TNF-α and IFN-γ, activating the CD4+ Th1 response. This activation of CD4+ Th1 during the acute phase of infection causes leukocyte adhesion to endothelial cells and accumulation at the site of inflammation resulting in decreased levels of circulating naïve, memory, and CD4+ Th1 T-lymphocytes (Mansueto et al. 2012). Interestingly, TNF-α expression remains higher than other cytokine levels despite the depletion of activated T-cells, which may be attributed to the presence of rickettsial eschars (de Sousa et al. 2007). For human rickettsioses, disease improvement is determined by the correlation of soluble TNF-α (sTNF-α) receptor and the level of TNF-α in the patient's serum (Kern et al. 1996). IFN-γ production by activated macrophages and NK cells is suppressed by IL-10, a Th2 cytokine. Elevated IL-10 levels in the circulatory system potentially fine tune the level of Th1 cytokines, thus preventing tissue damage (Capsoni et al. 1995). Elevated levels of IL-1, IL-6, TNF-α, and IFN-γ, as found in Balb/c mice during infection with *R. australis*, and elevated IL-6 levels during *R. conorii* infection of HUVECs support their roles as important immune effectors (Feng et al. 1993; Kaplanski et al. 1995).

3.3 Cell-Mediated Immune Response

CD8+ T lymphocytes, a subset of T lymphocytes, that the human host employs to combat intracellular bacterial infections, have two major functions: cytokine production and cytotoxic activity. CD8+ T lymphocytes recognize MHC class I molecules expressed on the surface of macrophages aiding in bacterial clearance. Secretion of IL-12 by APCs promotes clonal expansion of mature cytotoxic CD8+ T lymphocytes and contributes to rickettsial clearance, especially when compared to IFN-γ (Milano et al. 2000). MHC class I-deficient C57BL/6 mice are 50,000-fold more susceptible than wild-type C57BL/6 mice to *R. australis* infection. On the other hand, IFN-γ knockout mice are only 100-fold more susceptible to *R. australis* (Walker et al. 2001). These results were further supported by the administration of either mature (immune) CD8+ lymphocytes or naïve CD8+ lymphocytes to IFN-γ-knockout mice infected with *R. australis* and those receiving mature CD8+ lymphocytes were found to have lower rickettsial titers. Mice administered with mature CD8+ T lymphocytes also had significantly higher apoptotic cells in the liver than those administered with naïve CD8+ T lymphocytes. These findings suggest rickettsial clearance through the apoptotic mechanism of cytotoxic T-cells (Walker et al. 2001). While cytotoxic T lymphocytes clear rickettsial infections through the use of perforin, the roles of Fas-Fas ligand or granulysin have yet to be examined. CD8+ T-cells in cooperation with other immune cells, such as macrophages, NK cells, DCs, and CD4+ T-cells, contribute to immunity against *Rickettsia*. Infection of CD8-deficient mice with sublethal dose of *R. australis* can be detrimental to the host because of persistent infection leading to an adverse, fatal outcome (Li et al. 2003).

Regulatory T-cells (Treg), which maintain homeostasis by dominant negative regulation, are categorized into natural and adaptive regulatory T-cells based on the site of development. Natural Tregs develop within the thymus and are, as the name suggests, naturally $CD4^{+}CD25^{+}$. On the other hand, adaptive Tregs are naturally CD4+, but acquire CD25 expression in a location other than the thymus. Through interactions with DCs, *Rickettsia* may influence T-cell expansion by promoting the suppressive CD4+ T lymphocytes in susceptible hosts or the protective Th1 lymphocytes in resistant hosts (Fang et al. 2007). C3H/HeN mice infected with a lethal dose of *R. conorii* exhibit inhibited secretion of IFN-γ and IL-2, suppressed CD4+ T-cell production, and increased IL-10 expression due to the expression of a novel CD4+ $CD25^{+}$ $T\text{-}bet^{+}$ $Foxp3^{-}$ $CTLA\text{-}4^{high}$ suppressive Treg cell that produces IL-10 and IFN-γ. However, mice infected with a sublethal dose of *R. conorii* survive due to an induced protective immunity (Fang et al. 2009). $Foxp3^{+}$ Treg cell production requires IL-2, which is suppressed during the lethal dose thereby inhibiting $Foxp3^{+}$ expansion (Fang et al. 2009).

As chemokines function either in immune homeostasis or inflammation, they are critical in the clearance of bacterial infections. Certain chemokines on the surface of endothelial cells act as stop signals for T-lymphocyte migration through integrin affinity. Th1 and CD8+ T-cell specific chemokines and their receptors such as CCL2, CCL12 (CCR2, receptor for CCL2 and CCL12); CCL3, CCL4 (CCR5, receptor for CCL3 and CCL4); and CXCL9, CXCL10 (CX3CL1, receptor for CXCL9 and CXCL10) are highly expressed in the lungs of *R. conorii*-infected C3H/HeN mice. Expression of chemokine receptor CXCR3 and its ligands CXCL9 and CXCL10 helps in the recruitment of CD8+ T-cells and NK cells, required for bacterial clearance during the early phase of infection (Valbuena et al. 2003; Valbuena and Walker 2004).

3.4 Humoral Immune Response

Conventional wisdom supports the notion that *Rickettsia* are able to evade the humoral immune response due to the intracellular lifestyle. This argument is augmented by data suggesting that despite immunoglobulin therapy preinfection, nude mice still succumb to rickettsial infection. However, nude mice are either protected from infection or have a shorter recovery time when *Rickettsia* are pretreated with immune serum (Valbuena et al. 2002). The immunoglobulin therapy likely generates antibodies considered to be nonprotective, such as those against LPS, instead of yielding protective response such as that against outer membrane proteins. It may also be necessary to achieve a Th class switch in order to attain protection. Research has shown that antibodies against OmpA and OmpB protect C3H/HeN SCID mice from lethal doses of *R. conorii*, while antibodies against LPS fail to provide protection (Valbuena et al. 2002; Feng et al. 2004). The serum profile of patients infected with *R. africae,* the etiological agent of African tick bite fever, demonstrates high levels of IL-6 and reduced B lymphocyte levels. During later phases of rickettsiosis,

IL-10 inhibits IFN-γ and TNF-α production by Th1 cells. At this time, Th2 cytokines are secreted to promote B lymphocytes to produce immunoglobulins (Milano et al. 2000). IgM titers appear approximately 2 weeks after onset of clinical symptoms and persist until 4 weeks prior to decline. IgG levels also follow a similar trend, but remain high for months instead of declining as is the case for IgM (Fournier et al. 2002; Mansueto et al. 2012).

3.5 Regulation of Programmed Cell Death

Host–pathogen interplay and disease progression and outcome are determined by complex interactions and mechanisms involving defense responses on part of the host and evasion strategies employed by pathogens. Apoptosis is a host defense mechanism that can be manipulated and exploited by intracellular pathogens to ensure and promote their survival. For example, *Yersinia* and *Francisella* hijack apoptosis machinery for dissemination through the human host (Parandhaman and Narayanan 2014). Similarly, one would expect that apoptosis of *Rickettsia*-infected endothelial cells early during the course of infection will remove the supportive intracellular niche and interfere with bacterial multiplication and disease progression. Inhibition of NF-kB signaling pathway in endothelial cells infected with *R. rickettsii* results in the activation of upstream caspases 8 and 9 as well downstream, executioner caspase 3. This activation of apoptotic pathway leads to the cleavage of poly-(ADP-ribose)-polymerase, followed by DNA fragmentation. In addition, decrease in mitochondrial transmembrane potential causes cytochrome c release, which triggers the mitochondrial pathway of apoptosis via caspase 9 pathway. B-cell lymphoma-2 (Bcl-2) family proteins are mainly pro- and antiapoptotic factors, which are the key players in the programmed cell death by controlling mitochondrial permeability. In rickettsial infection, activation of NF-κB changes the expression of pro- and antiapoptotic proteins in favor of inhibition of apoptosis (Joshi et al. 2003, 2004; Chan and Yu 2004).

3.6 Rickettsial Motility

Used by other intracellular bacteria such as *Listeria* and *Shigella,* actin-based motility (ABM) is an active mechanism through which certain species of *Rickettsia* are able to move from cell to cell (Heinzen 2003). ABM requires the polymerization of actin filaments derived from the host's cytoskeleton to form the typical F-actin "comet tail." Acquisition of motility allows *Rickettsia* to move from cell to cell while avoiding the host's immune system. As early studies on the morphology of *Rickettsia* were conducted on fixed and stained tissues using light or electron microscopy, rickettsial motility remained undetected until live *R. rickettsii* were observed using phase contrast microscopy (Schaechter et al. 1957). With the

notable exception of *R. peacockii,* spotted fever group *Rickettsia* encode for actin-based motility. On the other hand, typhus group *Rickettsia* either do not encode for ABM, as is the case for *R. prowazekii*, or display an erratic pattern, for example *R. typhi* (Heinzen et al. 1993). This erratic motility is potentially caused by very short actin tails that appear to be hook shaped in morphology. No tail formation is, however, observed in both virulent and avirulent strains of *R. prowazekii* (Heinzen et al. 1993). For this reason, the primary method of dissemination within the host is through cell lysis. After cell invasion and escape from the phagosome, *R. prowazekii* replicates intracellularly every 8–9 h to numbers larger than 800 bacteria per cell with little to no cytopathic effect (Wisseman and Waddell 1975; Heinzen et al. 1993). This is in stark contrast to serious cytopathic effect noticed with approximately 100 spotted fever rickettsiae per cell. At a critical volume, the cell bursts releasing the typhus group rickettsiae into the extracellular milieu for invasion of other host cells (Heinzen et al. 1993).

For spotted fever group rickettsiae, polar actin tail formation was first observed within 30 min postinfection through dual fluorescence staining (Heinzen et al. 1993). For many years, this mechanism eluded experts. It was noticed early on that spotted fever group rickettsiae were the only group that could be seen in the nucleus as typhus group rickettsiae were only observed in the cytoplasm (Silverman and Wisseman 1979; Silverman et al. 1980). The mechanism was finally modeled after two other bacteria that also display actin-based motility. *Listeria monocytogenes* and *Shigella flexneri* lyse and escape the phagocytic vacuole, undergo cytoplasmic replication, and transport to other cells through actin filaments (Teysseire et al. 1992). Double fluorescence labeling of F-actin revealed comet-like structures extending from a polar end of *R. conorii*. Such actin tails were, however, observed very infrequently in *R. typhi*. Also, *R. conorii* were seen randomly dispersed throughout the cell, whereas *R. typhi* remained confined to the same location (Teysseire et al. 1992). Although *R. typhi* and *R. prowazekii* are both typhus group *Rickettsia*, *R. prowazekii* does not produce actin tails unlike the short tails of *R. typhi.* On the other hand, *R. rickettsii* has been demonstrated to utilize ABM within 15 min after host cell invasion (Heinzen et al. 1993). Since then, ABM has been shown in nearly all spotted fever rickettsiae except *R. peacockii,* which is considered to be nonpathogenic (Simser et al. 2002, 2005; Ogata et al. 2005; Serio et al. 2010). For *R. peacockii*, the lack of ABM is attributed to the insertion of a 1095 nucleotide ISRpe1 transposable element in the *rickA* gene among nine other locations within the genome (Simser et al. 2005).

RickA is a rickettsial surface protein that participates in actin polymerization through the activation of Arp2/3 (Gouin et al. 2004; Balraj et al. 2008b; Vellaiswamy et al. 2011a). A computational search of the *R. conorii* genome identified RC0909 to have similarity to *L. monocytogenes* ActA protein and both exhibited a weak similarity to the actin regulating Wiskott–Aldrich Syndrome Protein (WASP) homology domain 2 (Ogata et al. 2001). Since then, *rickA* has been identified either through bioinformatics analyses or experimentally in ancestral group rickettsiae (*R. bellii* and *R. canadensis*), transitional group rickettsiae (*R. felis* and *R. australis*), and spotted fever group rickettsiae (*R. africae, R. massiliae, R. monacencis, R. montanensis,*

R. raoultii, R. rickettsii, R. sibirica, and *R. slovaca*) (Heinzen et al. 1993; Jeng et al. 2004; Baldridge et al. 2005; Ogata et al. 2005, 2006; Balraj et al. 2008a, b). On the other hand, expression of *rickA* was not detected in typhus group rickettsiae, including *R. prowazekii* and *R. typhi* (Ogata et al. 2001; McLeod et al. 2004). Although the absence of *rickA* in *R. prowazekii* was not surprising considering that it lacks ABM, *R. typhi* demonstrates unique and erratic ABM, despite lacking a functional *rickA* (McLeod et al. 2004; Reed et al. 2014). Interestingly, time-lapse video demonstrated that *R. typhi* and *R. rickettsii* moved at the same rate (Heinzen 2003), suggesting the possible involvement of other actin-polymerization mechanisms. It was initially thought that *R. raoultii* was nonmotile as it formed 'microcolonies' in L929 (fibroblast) cells, which resembled *R. peacockii,* despite having a 565 aa protein homologous to RickA with conserved WASP-homology domains in addition to phospholipase D and hemolysin C proteins. However, upon infection of Vero cells (green monkey kidney epithelial cells), cell-to-cell spread was observed despite similar level of RickA expression in both L929 and Vero cells (Balraj et al. 2008a). Further, *R. canadensis* expressed RickA but did not undergo ABM (Heinzen et al. 1993). This suggests that RickA alone may not necessarily be sufficient or required for ABM. Nevertheless, it is clear that *rickA* originated early in rickettsial evolution and may have been lost during the divergence toward the typhus group.

Arp2/3 activation via ActA and IcsA has been implicated in ABM of *Listeria* and *Shigella,* respectively. The mechanisms between these bacteria and *Rickettsia* vary considerably, however. For example, *Listeria* and *Shigella* both form short, highly branched actin tails, whereas *Rickettsia* form long, unbranched tails (Cameron et al. 2001; Gouin et al. 2004). Further, immunofluorescence assays fail to detect Arp2/3 complex within the actin tail filaments of *R. conorii* despite being readily observable for *Shigella* (Gouin et al. 1999). *Shigella* requires the neuronal-WASP (N-WASP) signaling and Arp2/3 complex for ABM. Investigation of *R. rickettsii* actin tails, however, failed to show localization of N-WASP. Further, expression of verprolin, cofilin, and acidic domain (VCA) mutants completely inhibited *S. flexneri* ABM, yet had little effect on *R. rickettsii* and *R. parkerii* ABM. These results, thus, suggest that rickettsial ABM is independent of N-WASP and Arp2/3 complex (Harlander et al. 2003; Serio et al. 2010).

Recent evidence has suggested a role for Sca2 autotransporter in actin-based motility. A *sca2 R. rickettsii* mutant was created using the *mariner*-based transposon mutagenesis system and isolated using plaque purification. Since the plaques were visibly smaller than the parental *R. rickettsii* strain, actin tail formation was investigated. The mutant was found to no longer produce actin comet tails despite long actin tail formation for the parental strain. Further, the *sca2*-mutant failed to spread to nearby Vero cells as readily as the parental strain (Kleba et al. 2010). As a member of the surface cell antigen family, Sca2 was found to contain a C-terminal autotransporter domain and an N-terminal sec-dependent signal sequence (Blanc et al. 2005; Kleba et al. 2010; Sears et al. 2012). Computational analysis of *R. rickettsii sca2* revealed that it contains four WH2 domains and a proline-rich region sharing homology to formin homology 1 (FH1) domains, both of which are required for actin nucleation and polymerization (Kleba et al. 2010). Examination of other

Rickettsia species revealed that *R. prowazekii* completely lacks *sca2*, while *R. typhi* Sca2 lacks the FH1 domain and has the WH2 motifs in different locations with a divergence in sequences.

Recent research suggests that *Rickettsia* use both RickA and Sca2 for actin-based motility with two distinct phases. Previous research was performed under the assumption that one protein system (RickA vs Sca2) was used to activate ABM, similar to *Shigella* and *Listeria*. However, there are considerable differences between the organisms, such as replication time and persistence within the host cell. Initial studies examining rickettsial ABM were limited to early rickettsial events at the exclusion of later events (Reed et al. 2014). When early, intermediate, and late postinfection stages were compared, notable differences were found. As *R. parkeri* had already invaded host cells within 15 min, short or curved actin tails were predominant. However, at later times of 24–48 h postinfection, long tails were evident and more frequent than earlier time points. While actin polymerization was evident both early and the late during the infection, data suggest that early ABM is an Arp2/3-dependent mechanism similar to *L. monocytogenes* (Reed et al. 2014). Using the pMW1650/himar1 transposon system, *R. parkeri rickA* and *sca2* mutants were next isolated and purified. The *rickA* mutant showed no effect on ABM at later times, yet it was adversely affected for early actin comet tail formation. The *sca2* mutant displayed a reverse phenotype in that it had no adverse effect on the early actin tail formation but did have a defective tail formation during late stages (Reed et al. 2014). These data, thus, suggest that both Sca2 and RickA are needed to acquire actin-based motility, but Sca2 is important after the initial spread. Also, *sca2* mutant yielded significantly smaller plaques when compared to the wild-type *R. parkeri* strain and the *rickA* mutant. Further work is needed to understand why *Rickettsia* exploit more than one actin polymerization mechanisms despite a reductive evolution to eliminate unnecessary or redundant genes.

4 Secretion Systems

The secretion of rickettsial effectors remains an area of intense study. Similar to other bacteria, *Rickettsia* encode both Sec-dependent and Sec-independent secretory pathways (Fig. 19.8). Previous research using other bacterial pathogens demonstrated that most virulence factors were either secreted out of the cell into the environment or remained attached to the cell surface (Finlay and Falkow 1997). It is reasonable to expect that these pathways are critical for rickettsial survival within the host cells, but relatively little is known about their function(s).

Sec-dependent pathways include proteins that are secreted from the cytosol across the inner membrane into the periplasmic space. These proteins contain a transporter domain that is inserted into the outer membrane to facilitate their export. Transport across the inner membrane is accomplished through the SecYEG complex embedded in the bacterial inner membrane (Beckwith 2013). The Sec translocon is also composed of other integral membrane proteins, which include SecDF

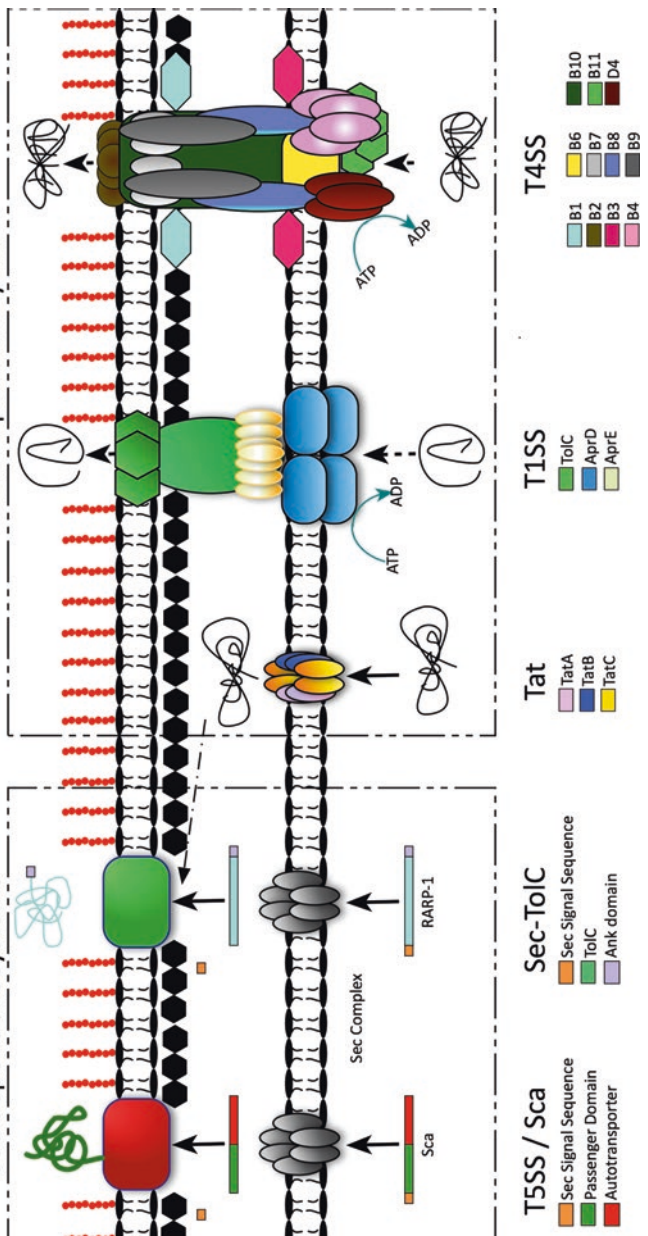

Fig. 19.8 *Rickettsia* secretion systems. Both Sec-dependent (*left*) and Sec-independent (*right*) secretion systems are encoded within the genome of *Rickettsia*. The type 5 secretion system (T5SS) comprise the surface cell antigen (sca) family, which includes OmpA and OmpB surface proteins. *Rickettsia* ankyrin repeat protein 1 (RARP-1), a known rickettsial effector, is secreted through the Sec-TolC system. The twin-arginine translocation (Tat) system, the type 1 secretion system (T1SS), and the type 4 secretion system (T4SS) are highly conserved throughout *Rickettsia*. Although not confirmed in *Rickettsia,* RalF is secreted through a T4SS in *Legionella*

complex, YajC, and YidC. In addition, the pathway also relies on other cytosolic proteins, namely, SecB and SecA, that prevent protein folding and help facilitate threading through the SecYEG complex, respectively (Beckwith 2013). An analysis of rickettsial genomes has revealed nearly all components of the Sec pathway (Ammerman et al. 2008; Gillespie et al. 2015). In vitro analyses using northern and western blotting have revealed expression of SecA in both *R. typhi* and *R. rickettsii* (Rahman et al. 2005). Transcription of *secA, secB, secD, secE, secF, secG,* and *secY* has also been reported during *R. typhi* infection of L929 cells (Dreher-Lesnick et al. 2008). The Sec-dependent pathway has two types of transporters: autotransporters and the Sec-TolC transporter. Within *Rickettsia*, the Sec-dependent autotransporters, also known as type V secretion systems, are composed of the *s*urface *c*ell *a*ntigens (Scas), discussed earlier in rickettsial adhesion to target cells.

The Sec-TolC translocon is a Sec-dependent pathway that utilizes the Sec complex to transport proteins from the cytosol to the periplasmic space. However, unlike the autotransporters, which contain the transporter domain, a TolC protein is embedded into the outer membrane to facilitate transport across the outer membrane. Expression of the TolC protein has been shown in *R. rickettsii* using immuno-electron microscopy and immunoblotting (Gong et al. 2014). Interestingly, *R. typhi* was also found to secrete an ankyrin repeat-containing protein, RARP-1, into host cells using the TolC pathway (Kaur et al. 2012). It was initially reported to be secreted through the type I secretion system, which uses a TolC protein, but further evidence suggested otherwise as the protein lacks a type I secretion system signal sequence. In addition, expression of both type I secretion system genes and RARP-1 showed no significant increase during infection, suggesting that RARP-1 secretion is independent of the type I secretion system (Gillespie et al. 2015). Nevertheless, further studies are required to gain a better understanding of type I secretion system of rickettsiae.

In addition to the Sec-dependent secretion systems, *Rickettsia* also encode several Sec-independent secretion systems. These include the twin-arginine-translocation (Tat) pathway, type I secretion system, and type IV secretion system. Unlike the Sec-dependent systems, these systems are known to export fully folded proteins. The Tat pathway was discovered more than 15 years ago in both eubacteria and archaea (Berks et al. 2000; Yuan et al. 2009). Despite advancements in the understanding of its molecular biology, the functions of the Tat pathway still remain unclear (Nunez et al. 2012). This pathway can transport proteins in their folded state across the cytoplasmic membrane, yet it does not cause the membrane to become permeable to protons and ions (Berks et al. 2000). The system is typically composed of five genes, *tatA-E*. However, these genes are separated into two locations within the bacterial chromosome. For *E. coli*, the *tatABCD* operon is located at 86 min, while *tatE* is located at 14 min (Sargent et al. 1998). These locations were determined using a classical interrupted mating experiment that maps the location of a gene as a function of time. Through a bioinformatics search, *Rickettsia* were found to encode for *tatA* and *tatC*, but lacked *tatB* (Nunez et al. 2012). Interestingly, although typically located in an operon, these two genes are located in different parts of the rickettsial genome. It is unclear whether reductive evolution or a rearrangement process caused this split (Berks et al. 2000; Nunez et al. 2012). Recently,

a BLASTp search of rickettsial genomes has revealed putative TatB proteins (Gillespie et al. 2015). Furthermore, computational approaches have identified a potential Tat signal peptide located on PetA, a ubiquinol-cytochrome c reductase, which has been shown to be translocated by the Tat pathway in plastids and other bacteria (Gillespie et al. 2015). It is currently unclear how folded proteins are transported from the periplasmic space through the outer membrane.

Unlike the Tat or Sec-dependent pathways, the type I secretion system (T1SS) spans from the inner membrane to the outer membrane and acts like a tunnel through the periplasmic space. It is divided into three parts: (1) inner membrane component (IMC), (2) membrane fusion protein (MFP), and (3) TolC. As the name suggests, the IMC is located within the inner membrane and has been identified as an ATP-binding cassette (ABC) transporter. The membrane fusion protein is a periplasmic adaptor protein that spans the periplasmic space and functions in substrate recognition. The TolC protein is embedded into the outer membrane as the final pore to the environment (Costa et al. 2015). The proposed mechanism of action for T1SS is that the IMC binds the substrate and transfers it to the MFP. Using ATP generated by the IMC, the MFP transfers it across the periplasmic space. The substrate binding recruits the TolC to the IMC–MFP complex, which completes the secretion process (Kanonenberg et al. 2013). The genes that code for a T1SS are found within all sequenced rickettsial genomes (Gillespie et al. 2008). Located adjacent to each other, *aprD* encodes for the rickettsial IMC and *aprE* encodes for the rickettsial MFP. Rickettsial T1SS effectors and their functions remain under investigation (Kaur et al. 2012; Gillespie et al. 2015). It is quite clear from the available literature, however, that studies of the rickettsial secretion systems have focused mostly on the type IV secretion system.

Found in both Gram-negative and Gram-positive bacteria, type IV secretion systems (T4SS) facilitate the secretion of proteins, DNA, and nucleoproteins across the bacterial membranes (Cascales and Christie 2003; Gillespie et al. 2009). The current classification recognizes three T4SS subfamilies: (1) conjugation systems (Lawley et al. 2003), (2) effector translocator systems (Blocker et al. 2003; Christie and Cascales 2005), and (3) DNA uptake and release systems (Dillard and Seifert 2001). However, previous classification schemes designated these T4SS types as F, P, and I based on the respective conjugative plasmids. These plasmids included IncF for plasmid F, IncP for plasmid RP4, and IncI for plasmid R64 (Lawley et al. 2003). Another classification scheme combined types F and P to create type IVA systems (VirB/VirD4) and plasmid IncI was renamed as type IVB (Dot/Icm) (Juhas et al. 2008). *Agrobacterium tumefaciens* has served as the model organism for investigations relating to VirB/VirD4 T4SS. Therefore, current information regarding T4SS function and structure has been determined using this model organism. The T4SS is divided into three subcomplexes: (1) energy subcomplex consisting of VirD4, VirB4, and VirB11; (2) inner membrane channel/core subcomplex consisting of VirB3, VirB6, VirB8, and VirB10; and (3) periplasmic/outer membrane channel composed of VirB1, VirB2, VirB5, VirB7, and VirB9 (Christie and Cascales 2005; Alvarez-Martinez and Christie 2009). Each component of the energy subcomplex contains a nucleotide triphosphate-binding site known as a Walker A site. Although these components carry the ability to bind ATP, only VirB11 can hydrolyze ATP. The

inner membrane subcomplex forms an inner membrane channel that serves as a scaffold for the periplasmic space T4SS proteins. The outer membrane subcomplex includes channel subunits that bridge across the periplasmic space and form the outer membrane channel. VirB2 subunit mainly creates the secretion channel and the T-pilus, while mutational studies with VirB5 subunit suggest its localization at the tip of the pilus (Fronzes et al. 2009). Analyses of sequenced rickettsial genomes have revealed the presence of type IVA T4SS with 11 scaffold proteins but lacking a *virB5* homolog (Gillespie et al. 2009, 2010). These 11 scaffold proteins are encoded by 18 genes. Interestingly, *virB4, virB8,* and *virB9* were duplicated as *virB4a* and *virB4b, virB8a* and *virB8b*, and *virB9a* and *virB9b*, respectively. A conserved RecA-like ATPase motif is only found on *virB4a* suggesting that *virB4b* is an ATPase mutant (Gillespie et al. 2009). *VirB9a* contains a full-length C-terminal domain unlike *virB9b,* which has a truncated C-terminal domain. *VirB8a* has a mutation in the critical C-terminal region, NPxG, likely rendering it nonfunctional (Gillespie et al. 2009). Further, there are five copies of *virB6—virB6a, virB6b, virB6c, virB6d,* and *virB6e.* Although the VirB6 family as such is highly conserved, the genes demonstrate significant divergence from one another (Gillespie et al. 2015). Currently, their functions are unknown, but there is evidence in *R. typhi* to suggest that they serve as a surface structure (Sears et al. 2012). On the other hand, *virB1, virB2, virB3, virB7, virB10, virB11,* and *virD4* have one copy of their respective genes and are highly conserved across the genus. Similar to *Bordatella pertussis,* which also lacks a *virB5* gene, *Rickettsia* most likely does not form a T-pilus. Considering the obligately intracellular lifestyle of *Rickettsia,* it most likely deposits the secreted proteins directly into the host cytoplasm. This would suggest that a *virB5* is unnecessary, as an extended pilus would not be required to deposit substrates into the host cytoplasm. It remains unclear whether the T4SS is truly active, as no substrates have yet been identified. Recently, RalF, encoded by *R. prowazekii* and *R. typhi* was hypothesized to be a possible rickettsial effector. RalF is a Sec7 domain protein known only from *Rickettsia* and *Legionella* (Cox et al. 2004; Rennoll-Bankert et al. 2015). Interestingly, *Legionella pneumophilia* RalF (LpRalF) and *R. prowazekii* (RpRalF) are strikingly similar despite these bacteria being distantly related. The RalF protein functions as a guanine nucleotide exchange factor (GEF) of ADP ribosylation factors (Arfs) in eukaryotes (Casanova 2007). LpRalF is a secreted effector of the Dot/Icm T4SS (Nagai et al. 2005). It was shown recently in *R. typhi* that RalF is expressed early during infection and diminished after internalization into the host cell. A bacterial two-hybrid assay demonstrated interaction between RalF and VirD4 suggesting that *R. typhi* secreted RalF during infection. Arf6 and $PI(4,5)P_2$ recruitment by RalF was necessary for *R. typhi* invasion of host cells (Rennoll-Bankert et al. 2015). Further, immunofluorescence assays demonstrated localization of RalF from *R. typhi, R. felis,* and *R. montanensis* on the bacterial membrane. Interestingly, for *R. bellii*, RalF was localized to the host endoplasmic reticulum. When *R. bellii* RalF was compared to all other *Legionella* and *Rickettsia* RalFs, three conserved rickettsial insertion sequences in the Sec7-capping domain (SCD), a domain that regulates RalF interaction with Arfs, were found to be missing, but a unique insertion sequence and a slightly less hydrophobic active site were

present (Amor et al. 2005; Rennoll-Bankert et al. 2015). The significance of these unique features remains unknown. However, the localization of RalF to the endoplasmic reticulum, as opposed to the bacterial membrane, may be the result of its association with amoebas (Ogata et al. 2006; Rennoll-Bankert et al. 2015).

5 Metabolism

Rickettsia are strictly intracellular bacteria and as such have undergone strong reductive evolution (Andersson et al. 1998). The elimination of nonessential or superfluous genes has resulted in a genome that requires a tight association with the host. Transporter systems seem to have replaced the synthetic pathways, imparting the ability to acquire energy and metabolites from nutrient rich cytosol of the host cell (Renesto et al. 2005). For this reason, the nutritional requirements of *Rickettsia* are critical for understanding their virulence and pathogenesis.

As with all living organisms, ATP is the primary source of energy for rickettsial growth and replication. Due to diverse environmental conditions encountered as part of their lifecycles, bacteria have evolved numerous methods to synthesize ATP. For aerobic conditions, a common method of respiration is glycolysis and oxidative phosphorylation (Poole and Cook 2000). For most genera of the order *Rickettisales*, the glycolytic pathway is complete, but interestingly, analyses of the *R. prowazekii* and *R. conorii* genomes revealed that neither has retained genes for the glycolytic enzymes, thus making it nonfunctional within *Rickettsia*. In addition, both rickettsial species were shown to have eliminated the genes required for nucleotide or cofactor biosynthesis (Dunning Hotopp et al. 2006). Sequencing of other rickettsial genomes has revealed this to be true for other species (Fuxelius et al. 2007). *Orientia,* which is closely related to *Rickettsia*, has retained three glycolytic genes (*tpiA, gap,* and *pgk*). Despite not encoding for a glycolytic pathway, the ATP production profile for *Rickettsia* and mitochondria is strikingly similar, as they both possess a high number of ATP/ADP translocases, suggesting a common ancestry (Andersson et al. 1998; Renesto et al. 2005). As obligately intracellular organisms, rickettsiae effectively exchange host ATP for ADP until the supply is limited. At this point, the bacteria produce their own ATP as the sole energy source. This is attributed to their retaining of the required genes for the tricarboxylic acid cycle (Krebs cycle) and an electron transport chain (Frohlich and Audia 2013). As such, *Rickettsia* can create a proton motive force to drive oxidative phosphorylation for the *de novo* production of ATP.

Not only are *Rickettsia* missing the glycolytic pathway for energy synthesis, all species lack nucleotide metabolism essential for not only energy but also for RNA and DNA production. *Rickettsia* are unable to synthesize both purines and pyrimidines as the genes for their biosynthetic pathways have been eliminated (Andersson et al. 1998). The only purine synthesis pathway gene encoded in *R. prowazekii* is 5-aminoimidazole-4-*N*-succinylcarboxamide ribonucleotide (*purC*). Nevertheless, the genome does encode several proteins that allow for the scavenging of purines

from the host environment (Renesto et al. 2005). Further, it encodes four genes that are required for the conversion of nucleoside monophosphates into nucleotide diphosphates (*adk, gmk, cmk,* and *pyrH*); two genes for converting ribonucleoside diphosphates to deoxyribonucleoside diphosphates; and one gene, *ndk*, for conversion of NDPs and dNDPs to NTPs and dNTPs (Andersson et al. 1998). While *Rickettsia* may not produce their own nucleosides, they can convert nucleoside monophosphates acquired from the host into other nucleotides.

Both eukaryotic and prokaryotic organisms rely on the use of coenzymes and cofactors to facilitate enzymatic reactions. Cofactors include metal ions such as Cu^{2+}, Fe^{2+}, Fe^{3+}, Zn^{2+}, and Na^{+}; coenzymes are organic small molecules such as NAD+, NADP+, FAD+, and biotin. It was originally thought that the rickettsial membrane was "leaky" to certain cofactors and coenzymes as they lack the ability to synthesize nicotinate and nicotinamine, the precursors to NAD+ and NADP+ (Atkinson and Winkler 1989). However, it was later found that *Rickettsia* rather encode transporters for large and charged molecules such as NAD+. Yet, *R. prowazekii* was unable to transport intact, radiolabeled NAD+. Despite this, *R. prowazekii* encodes NAD+ kinase and nictoinamide nucleotide transhydrogenase to facilitate interconversion between NAD+ and NADP+ (Renesto et al. 2005). The exact mechanism for NAD+ and NADP+ synthesis in rickettsiae still remains elusive.

Sequencing of *R. prowazekii* genome also revealed evidence for the lack of amino acid metabolism (Andersson et al. 1998; Fuxelius et al. 2007). For example, the enzymes required for glutamate metabolism were found to be eliminated with the exception of aspartate amino-transferase A (Renesto et al. 2005). Glutamine is the precursor to amino acid synthesis, purine and pyrimidine synthesis for nucleotides, and acts as a carbon source for oxidation (Newsholme et al. 2003). *Rickettsia prowazekii* growth in Vero cells is compromised unless serine and glycine are supplemented into the culture medium. Accordingly, serine and glycine levels are 31 and 14 % higher in mock-infected cells in comparison to *R. prowazekii*-infected Vero cells. These data suggest the reliance of *R. prowazekii* on the host's serine and glycine pools for growth and survival (Austin et al. 1987). Similarly, the enzymes for aspartate and alanine metabolic pathways are also missing in *Rickettsia* species. In fact, since alanine aminotransferase is missing from the genome, it is thought that the conversion of L-alanine to D-alanine, a major component of peptidoglycan, is performed through the alanine racemase gene, *alr* (Renesto et al. 2005). The biosynthetic pathways for branched-chain amino acids, which include valine, leucine, and isoleucine, and the aromatic amino acids (tryptophan, tyrosine, and phenylalanine) are also absent in *Rickettsia*. It is, thus, clear that *Rickettsia* must rely on the import of host amino acids for their growth, survival, and replication.

6 Genomics

In 1998, the chromosome of *R. prowazekii* strain Madrid E became the first rickettsial genome to be fully sequenced and published (Andersson et al. 1998). An examination of the genome revealed a pattern consistent with reductive evolution

as *Rickettsia* continue to purge unnecessary and redundant genes to adapt to the obligately intracellular lifestyle. Many of the genes required by free-living bacteria are absent in *Rickettsia,* such as the biosynthetic pathways discussed earlier. Further, nearly 150 nucleus-encoded mitochondrial proteins are homologous to proteins found in *R. prowazekii,* with significant similarity in transport mechanisms and ATP production associated with the TCA cycle and respiratory chain complexes. Once encoded in the mitochondrial ancestor genome, nucleus-encoded mitochondrial proteins help to control mitochondrial functions, but are now encoded in the nuclear DNA. Data suggest that the origin of aerobic respiration may have evolved from an ancestor of *Rickettsia*, which possibly diverged approximately 1.5–2 billion years ago (Andersson et al. 1998), followed by transition of a free-living *Rickettsiales* ancestor to the obligately intracellular lifestyle between 775 and 525 million years ago (Merhej and Raoult 2011). *Rickettsiales* association with arthropods did not occur until the Cambrian explosion, in which arthropods first evolved approximately 542–500 million years ago. The genus known today as *Rickettsia* did not appear until the evolution of the rickettsial hydra group 150 million years ago. A rapid radiation of rickettsial groups occurred 50 million years ago bringing about the ancestors to the rickettsial groups known today (Weinert et al. 2009b).

The genus *Rickettsia* has a genome size ranging from 1.11 to 2.1 Mb (Table 19.3). The genomes of spotted fever group *Rickettsia* range from 1.27 to 2.1 Mb, while typhus group is approximately 1.11 Mb. Ancestral group *Rickettsia* and transitional group *Rickettsia* range from 1.15 to 1.53 Mb and 1.23 to 1.59 Mb, respectively (Merhej et al. 2014). Surprisingly, rickettsial genomes are rather AT-rich with GC content ranging from 28.9 % in typhus group to 33.3 % in the spotted fever group. *Rickettsia endosymbiont of Ixodes scapularis* (REIS) has both the largest genome and the highest GC content of any *Rickettsia*. Typhus group rickettsiae have the least number of annotated open reading frames (ORFs) and proteins encoded in their genomes, 865–999 and 829–950, respectively. In the course of switching from a free-living to an obligately intracellular bacterium, data suggest that *Rickettsia* have lost about 2135 genes due to reductive evolution (Georgiades et al. 2011; Georgiades and Raoult 2011; Merhej and Raoult 2011). Interestingly, *Rickettsia* has a high percentage of noncoding DNA when compared to other bacteria. *Rickettsia prowazekii* and *R. conorii* contain 24 and 19 % noncoding DNA, respectively, yet *Chlamydia trachomatis*, another obligately intracellular bacterium, contains only 10 % noncoding DNA (Andersson et al. 1998; Holste et al. 2000; Rogozin et al. 2002). *Carsonella ruddii*, an endosymbiont of psyllids, encodes for the smallest known genome with 182 ORFs and 97.3 % coding density (Nakabachi et al. 2006).

Noncoding DNA in rickettsial genomes is traditionally considered as merely "junk DNA" resulting from gene degradation. The existence and expression of bacterial noncoding RNAs in intergenic regions has been well documented and their function(s) have been characterized. These noncoding RNAs have transcriptional regulatory functions in virulence, metabolism, stress response, antibiotic resistance, and quorum sensing (Narberhaus and Vogel 2009; Waters and Storz 2009; Lalaouna et al. 2014). Further, noncoding RNAs have been documented in bacteria

Table 19.3 Rickettsial genomes and associated plasmids

Rickettsial group	Species	Strain	Genome size (bp)	GC%	CDS	Plasmid
Ancestral group						
	R. bellii					
		RML 369-C	1,552,076	31.6	1429	–
		OSU 85-389	1,528,980	31.6	1476	–
	R. canadensis	McKiel	1,159,772	31.1	1093	–
Typhus group						
	R. prowazekii					
		Madrid E	1,111,523	29	835	–
		Rp22	1,111,612	29	952	–
		Breinl	1,109,301	29	920	–
	R. typhi	Wilmington	1,111,496	28.9	838	–
Transitional group						
	R. akari	Hartford	1,231,060	32.3	1259	–
	R. australis					
		Cutlack	1,323,280	32.3	1261	pRau
		Phillips	1,320,570	32.2	1715	–
	R. felis	URRWXCal2	1,485,147	32.5	1512	pRfe pRfel1
Spotted fever group						
	Candensis R. amblyommii		1,448,020	32.4	1821	pRam18 pRam23 pRam32
	R. africae	ESF-5	1,278,540	32.4	1041	pRaf
	R. conorii	Malish 7	1,268,755	32.4	1374	–
	R. heilongjiangensis	54	1,278,471	32.3	1297	–
	R. helvetica	C9P9	1,369,827	32.2	1739	pRhe
	R. honei	RB	1,268,760	32.4	1614	–
	R. japonica	YH	1,279,890	32.4	971	–

	R. massiliae					
		MTU5	1,376,180	32.5	980	pRma
		AZT80	1,278,720	32.6	1207	pRmaB
	R. monacensis	IrR/Munich	1,353,450	32.4	1460	IrR/Munich
	R. montanensis	OSU 85	1,279,798	32.6	1217	–
	R. parkerii	Portsmouth	1,300,386	32.4	1318	–
	R. peacockii	Rustic	1,288,492	32.6	947	pRpe
	R. philipii	364D	1,287,740	32.5	1344	–
	R. raoultii					pRra1 pRra2 pRra3
	R. rhipicephali					
		3-7-female6-CWPP	1,305,470	32.4	1266	pRrh
		HJ#5	1,448,630	32.3	1255	–
		Ect	1,266,920	32.6	1563	–
	R. rickettsii					
		Sheila Smith	1,257,710	32.5	1345	–
		Iowa	1,268,201	32.4	1384	–
		R	1,257,005	32.5	1334	–
		Brazil	1,255,681	32.5	1332	–
	R. sibrica					
		mongolitimonae	1,252,340	32.4	1616	–
		sibirica BJ-90	1,254,730	32.5	1588	–
	R. slovaca					
		13-B	1,275,090	32.5	1112	–
		D-CWPP	1,275,720	32.5	1347	–
	REIS		2,096,878	33	2117	pReis1 pReis2 pReis3 pReis4

belonging to Enterobacteriaceae, *Listeria monocytogenes, Clostridium perfringens, Staphylococcus aureus, Pseudomonas aeruginosa,* and *Mycobacterium tuberculosis* (Papenfort and Vanderpool 2015). Bacterial noncoding RNAs are generally 50–500 nucleotides in length and originate from two scenarios: intergenic regions (trans-acting) and antisense to an ORF (cis-acting). *Rickettsia prowazekii* termination sites have been shown to possess stem loop structures after homopolymeric poly(T) stretches (Woodard and Wood 2011). The leaky termination sites in *R. prowazekii* create antisense RNAs, which may be responsible for posttranscriptional regulation. Recently, 1700 small RNAs (sRNAs) were predicted using bioinformatics analyses of 16 genomes representing 13 rickettsial species belonging to all rickettsial groups (Schroeder et al. 2015). Predicted sRNAs from *R. prowazekii, R. typhi, R. rickettsii,* and *R. conorii* were analyzed for σ^{70} promoters and potential targets predicted for the virulent *R. prowazekii* strain Breinl. A total of 26 candidate sRNAs were experimentally verified using both next-generation sequencing and RT-PCR. To determine independent expression of sRNA candidates, the read coverage for each nucleotide was normalized to the length spanning the potential sRNA length and the mean expression value (MEV) was calculated by determining the ratio between the sRNA and the flanking 50-nucleotide positions. Twelve candidates were found to have an MEV >1.5, suggesting independent expression. Of these, six candidate sRNAs were further verified using RT-PCR, presenting clear evidence for the expression of noncoding RNAs within the *R. prowazekii* (Schroeder et al. 2015). However, a limitation of this study was the potential for identifying only trans-acting sRNAs and excluding any potential cis-acting sRNAs. The posttranscriptional regulation of rickettsial genes via small RNAs is a fascinating new area for rickettsial gene regulation.

Due to the obligately intracellular lifestyle of rickettsiae, mobile genetic elements were long considered to be uncommon. Today, at least 11 *Rickettsia* species have been found to contain plasmids with diversity among the number carried by different species/strains (El Karkouri et al. 2016). These 11 species, representing the transitional group (*R. australis* and *R. felis*) and the spotted fever group (*R. africae, R. amblyommii, R. helvetica, R. massiliae, R. monacensis, R. peacockii, R. raoultii, and R. rhipicephali*) harbor at least 20 known rickettsial plasmids (Table 19.3). It is important to consider, however, that nine spotted fever group, one transitional group, and two typhus group *Rickettsia* species have no detectable plasmid. These species include *R. akari, R. conorii, R. japonica, R. montanensis, R. parkeri, R. philipii, R. rickettsii, R. sibirica, R. slovaca, R. prowazekii, R. typhi,* and *R. canadensis* (El Karkouri et al. 2016). The first rickettsial plasmid, pRF, was discovered after the sequencing of the *R. felis* genome. Although two plasmids pRF and pRFδ, were initially described, pRFδ is mostly likely an artifact of the genome assembly for a number of reasons that include the absence of required maintenance genes and failure to amplify in other strains (Gillespie et al. 2007). However, the 62.8 kb pRF plasmid contains 68 ORFs encoded within its sequence. Upon sequencing the REIS genome, four novel plasmids (pREIS1 through pREIS4) were identified. Interestingly, 72 % of the ORFs encoded on the REIS plasmids were entirely novel

as they were not found in another *Rickettsia* species. pREIS2 is the largest known rickettsial plasmid at 66.8 kb with 83 potential ORFs of which 82 % are functionally annotated and 18 % encode hypothetical proteins (Gillespie et al. 2012). In addition to the plasmids, the REIS genome was found to be overrun with more than 650 transposons, mobile genetic elements, and amplified genetic elements. This suggests that rickettsial genes associated with its intracellular lifestyle were acquired via gene transfer (Gillespie et al. 2012). Interestingly, analysis of plasmid gene content exhibiting 16–59 % gene degradation suggests that rickettsial plasmids are also undergoing reductive evolution similar to that seen on chromosomes (El Karkouri et al. 2016).

Rickettsial plasmids and mobile genetic elements highlight the importance of horizontal gene transfer (HGT). Although first described in the 1940s, HGT is a critical evolutionary mechanism that ensures continued adaptation to new environments by conjugation, transformation, or transduction (Sahni et al. 2013; Soucy et al. 2015). Previous research into rickettsial evolution placed only scant emphasis on rickettsial HGT due to its intracellular lifestyle. Nevertheless, a focus on HGT and its role in rickettsial evolution after genome sequencing has continuously demonstrated mobile genetic elements (Ogata et al. 2006; Gillespie et al. 2007, 2012; Weinert et al. 2009a; Merhej et al. 2011; Merhej and Raoult 2011; El Karkouri et al. 2016). To determine the origin of rickettsial plasmids, an examination of 260 plasmid genes was conducted. Vertical transmission of the last common plasmid ancestor, labeled pRICO, was partially responsible for the current set of rickettsial plasmids. The plasmids, despite undergoing gene loss resulting in reductive evolution, were successful in gaining new genes via HGT and by numerous duplication events. Although plasmids gained genes from HGT, the degradation of genes probably resulted in permanent plasmid loss noted in several rickettsial species (El Karkouri et al. 2016). Evidence of such HGT has been demonstrated by the observation of pili between two rickettsial bacteria. For *R. felis,* two pili were observed by transmission electron microscopy on the surface of the bacteria. These include pili that are conjugative in nature and establish direct contact with other bacteria and short hair-like pili that are most likely involved in bacterial attachment to host cells (Ogata et al. 2005). Shortly after observation with *R. felis*, pili formation was noted in *R. bellii.* A *tra* gene cluster conserved in the *R. bellii* genome sequence supports its ability to form pili. *Tra* genes are composed of an F-like and a Ti-like region (Ogata et al. 2006). The F-like region encodes 12 conjugative transfer proteins, which correspond to the T4SS core proteins and auxiliary proteins (Lawley et al. 2003). The Ti-like region encodes the TraA ORF, responsible for the activity of nickase and helicase in initiating DNA transfer, and TraD, which has an unknown function. Interestingly, the incongruence in *R. bellii* gene phylogenies indicates the occurrence of HGT between *R. bellii* and other bacterial species including *R. typhi*, *Legionella* spp., *Francisella* spp., and *Burholderia* spp. It is, therefore, likely that the hosts had coinfections with these bacteria and *R. bellii* resulting in HGT (Merhej et al. 2011).

7 Conclusions and Future Directions

Vector-borne rickettsioses remain a global health concern and the clinical diagnosis of rickettsial infections is often difficult owing to common flu-like symptoms such as nausea, headache, fever, and vomiting, which are shared by several other pathogens. This often results in the use of inappropriate treatment regimens during early stages of infection when antibiotics are effective, thus resulting in potentially severe complications and fatal infections leading to >20 % mortality. The development of rapid, reliable, and cost-effective molecular diagnostics is warranted in place of current strategies that rely on serological testing, indirect immunofluorescence assays, and/or immunohistochemistry of skin biopsies. Several recent studies have shown rodents and household pets as potential reservoirs resulting in endemic outbreaks (Hii et al. 2011; Levin et al. 2011; Day et al. 2012). Vector control through eradication of reservoir hosts, especially rodents, should be focused upon for limiting rickettsial transmission. Though not yet well established, a role for vector gut microbiome is implicated in pathogen uptake and maintenance in arthropod vectors (Dennison et al. 2014). *Rickettsia* are predominantly transmitted by ticks, fleas, and lice, and depending on the geographic location, a majority of *Rickettsia* species are now known to be transmitted by more than one vector species. Despite over 100 years of research, an effective rickettsial vaccine that could provide a stable and lifelong immunity has yet to be developed. Though recombinant vaccines against OmpA and OmpB have been tested for protection against a few rickettsial species, development of a live attenuated vaccine will be helpful, as it is likely to provide better protection in comparison to subunit vaccines (Walker 2009).

Rickettsia primarily target the endothelial lining of the blood vessels. However, the mechanisms by which *Rickettsia* enter and escape phagosomal degradation in endothelial cells are not completely clear. To date, only three host cell receptors have been implicated in facilitating rickettsial adhesion and entry (Martinez et al. 2005; Gong et al. 2013; Hillman et al. 2013). It is hypothesized that *Rickettsia* employ multiple host receptors to gain entry. Uncharacterized proteases encoded by rickettsial genomes may facilitate their escape from phagosomes leading to establishment of intracellular infection. Interestingly, although a role for pattern recognition receptors and inflammasomes, such as TLR2, TLR4, and NLRC3, has been implicated in immune activation during rickettsioses, the role of cytosolic receptors such as NOD1 and NOD2 is yet to be determined. It is possible that rickettsial cell wall components and effector molecules such as RARP1, RalF, and VapC, which are secreted into the host cytosol, may play a role in immune modulation. Bacterial ankyrin repeat containing proteins (Anks) are also secreted into the host cytosol. In *Ehrlichia*, Anks are shown to be translocated into the host nucleus and regulate host gene expression (Zhu et al. 2009, 2011). Though rickettsial genomes encode for several Anks, their role in pathogenesis is not clear. In this regard, future studies focused on understanding the roles of *Rickettsia* encoded proteins in the modulation of host transcriptome and immune evasion is expected to shed light on their survival strategies.

Rickettsial genomes are recently shown to harbor posttranscriptional regulators that are potentially involved in transcriptome regulation during host–pathogen and vector–pathogen interactions (Schroeder et al. 2015). Additionally, the role for HGT is now well appreciated to shape bacterial genomes and nearly 30–40 % of the ORFs in rickettsial genomes encode for hypothetical proteins, a majority of which are ORFans (genes with no detectable homologies in known databases). It is quite likely that a number of posttranscriptional regulators and hypothetical proteins are critical for rickettsial survival, transmission, and pathogenesis, and further studies addressing these aspects should provide novel insights into the pathogenesis of these fascinating intracellular pathogens.

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Chapter 20
Genomes of *Rickettsia* Species

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1 Introduction

The strictly intracellular lifestyle of rickettsiae, and thus the few phenotypic characters they express, made traditional identification methods used in bacteriology unapplicable. As a consequence, the word "rickettsia" has long served as a generic term for most of small and uncultivable bacteria that were not otherwise identified. However, the introduction of molecular identification techniques in the past three decades has revolutionized the taxonomy of "rickettsiae". Consequently, the term "rickettsia" currently only applies to arthropod-borne bacteria belonging to the genus *Rickettsia* within the family *Rickettsiaceae* (order *Rickettsiales* α-*Proteobacteria* class). Bacteria within the genus *Rickettsia* are obligate intracellular, short rods that retain basic fuchsin when stained by the Gimenez method, grow in strict association with eukaryotic cells within which they live free, divide by binary fission in the cytoplasm, and may cause diseases in invertebrate hosts (which act as vectors and reservoirs) or vertebrate hosts (Parola et al. 2013).

Although initially discovered in the early twentieth century, *Rickettsia* species have remained poorly known until the 1990s and the introduction of molecular tools. Currently, the *Rickettsia* genus contains 27 officially validated species (Fig. 20.1, http://www.bacterio.net/rickettsia.html), and several dozens of as-yet uncharacterized strains or arthropod-amplicons. The main vectors of these bacteria are ticks, which are also their reservoirs, but some are associated with lice, fleas or mites. Some members of the genus *Rickettsia* are recognized human pathogens, but the others should rather be considered as species or strains of unknown pathogenicity than as non-pathogenic, especially when associated with arthropods able to bite humans.

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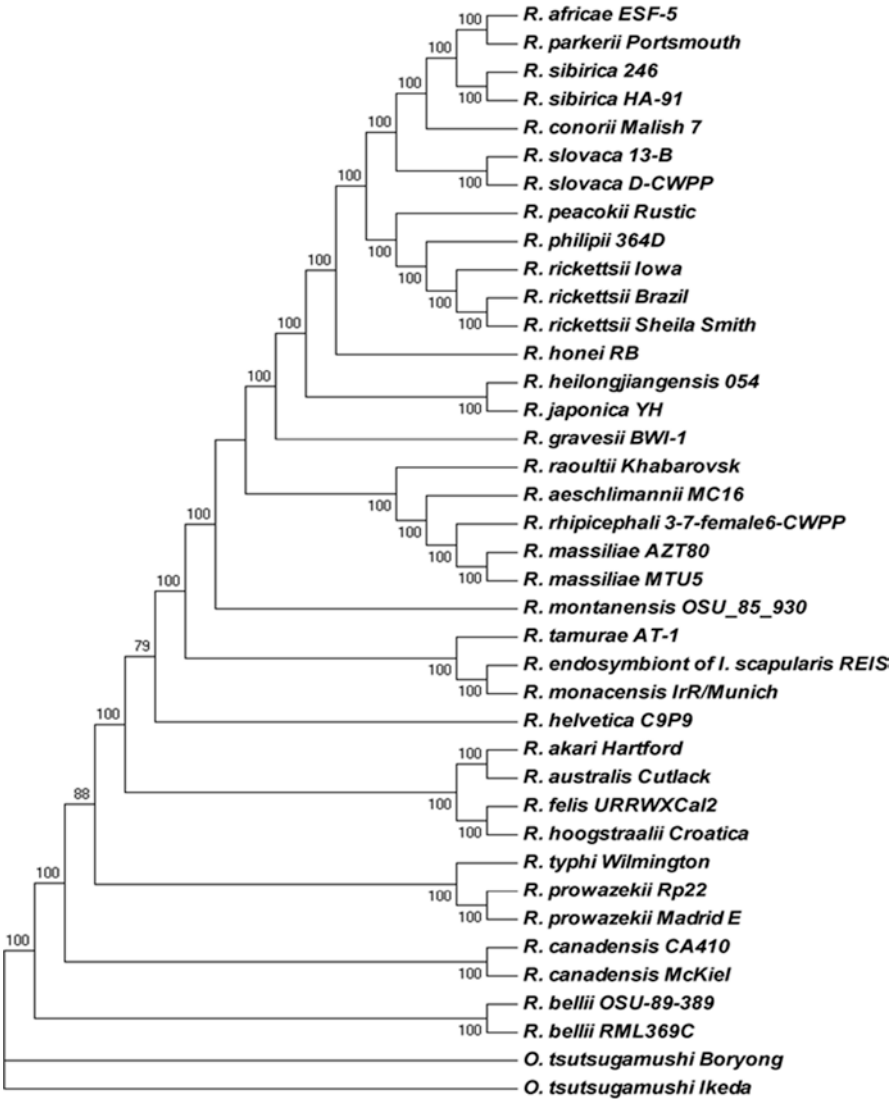

Fig. 20.1 Phylogenomic tree of 29 *Rickettsia* species based on the sequences of 330 core proteins using the Maximum Likelihood method with GAMMA and JTT models and display only topology. Values at the nodes are percentages. Numbers at nodes are percentages of bootstrap values obtained by repeating analysis 200 times to generate a majority consensus tree. Only values higher than 70 % were reported. *Orientia tsutsugamushi* was used as outgroup

Rickettsia species may cause rickettsioses, which are amongst the oldest known arthropod-borne diseases. To date, 17 rickettsioses have been identified. The pathogenicity of members of the *Rickettsia* genus as well as other phenotypic properties has been the basis for their classification into two main groups, i.e. the spotted fever and typhus groups: (1) spotted fever group rickettsiae are mainly associated with

ticks, cause spotted fevers in humans, have an optimal growth temperature of 32 °C, a G+C content between 32 and 33, can polymerize actin and thus move into the nuclei of host cells; (2) in contrast, typhus group rickettsiae are associated with human body lice (*R. prowazekii*) or fleas (*R. typhi*), cause epidemic or murine typhus, have an optimal growth temperature of 35 °C, a G+C content of 29 %, cannot polymerize actin and thus cannot enter the nuclei of host cells and are only found in their cytoplasm. In addition, *R. bellii* and *R. canadensis*, two species of unknown pathogenicity, were classified in the ancestral group, and *R. akari*, transmitted by mites and the agent of rickettsial pox, *R. australis*, associated to ticks and causing Australian tick typhus, and *R. felis*, transmitted to humans by fleas and mosquitoes and causing flea borne rickettsiosis, were proposed to belong to an uncertain "transitional group" (Gillespie et al. 2015a). *Rickettsia* species possess major antigens against which infected humans produce antibodies. These include the lipopolysaccharide (LPS) and outer membrane proteins of the surface cell antigen (SCA) family, mostly rOmpB (or Sca5), rOmpA (or Sca0, only present in spotted fever group rickettsiae), and Sca4.

2 Main Genomic Characteristics of *Rickettsia* Species

The genomics era started in 1995 with the complete sequencing of the *Haemophilus influenzae* and *Mycoplasma genitalium* genomes (Fleischmann et al. 1995; Fraser et al. 1995). Genome sequencing soon appeared as a powerful method to uncover bacterial properties, especially those of fastidious bacteria. The first rickettsial genome sequenced was that of *R. prowazekii*, in 1998 (the 13th published bacterial genome) (Andersson et al. 1998). This first genome contained only 843 genes for a size of 1.1 Mb (Table 20.1). The small gene content is explained by the high proportion (24 %) of non-coding DNA. Many genes involved in the biosynthesis and regulation of biosynthesis of amino acids and nucleosides in free living bacteria were absent, which prompted the authors to compare the *R. prowazekii* genome to that of mitochondria.

In 2001, the sequencing of the genome from *R. conorii*, the agent of Mediterranean spotted fever, allowed the first rickettsial genome comparison (Ogata et al. 2001). This study provided new insights into the evolution of *Rickettsia* species, by identifying in the *R. conorii* genome the presence of an additional 514 open reading frames (ORFs) and a tenfold increase in the number of repetitive elements named rickettsia palindromic elements (RPEs, 95- to 150-bp long), for a size difference of only 0.15 Mb. In the *R. conorii* genome, a total of 656 RPEs organized in 10 families were identified, representing 3.2 % of the entire genome (Ogata et al. 2002). Unexpectedly, some of these RPEs were found inserted into protein-coding genes within which they were compatible with the three-dimensional fold and functions of the encoded proteins (Ogata et al. 2002). The *R. prowazekii* and *R. conorii* genomes also exhibited 5S and 23S rRNA genes separated from the 16S rRNA gene, and were almost perfectly colinear, two properties later confirmed for most *Rickettsia* genomes.

Table 20.1 Main features of *Rickettia* genomes available in GenBank

Species	Strain	Genome size (Mb)	G+C content (%)	Protein-coding genes	Presence of plasmid	Accession number
R. aeschlimanni	MC16	1.31	32.2	1051	Plasmid 1, Plasmid 2	CCER01000000
R. africae	ESF-5	1.28	32.4	1219	pRaf	NC_012633
R. akari	Hartford	1.23	32.3	1259		CP000847
"*R. amblyommii*"[a]	Ac37	1.46	32.4	1511	pRAMAC18, pRAMAC23	CP012420
"*R. amblyommii*"[a]	GAT-30V	1.48	32.4	1550	pMCE1, pMCE2, pMCE3	CP003334
"*R. amblyommii*"[a]	AC/PA	1.44	32.4	1123		LANR00000000
"*R. amblyommii*"[a]	Darkwater	1.44	32.8	1060		LAOH00000000
R. argasii	T170-B	1.44	32.4	1187		LAOQ00000000
R. australis	Cutlack	1.33	32.3	1136	pMC5_1	NC_017058
R. australis	Phillips	1.32	32.2	1099	pRau01	AKVZ00000000
R. bellii	RML369-C	1.52	31.7	1429		NC_007940
R. bellii	OSU 85-389	1.52	31.6	1476		NC_009883
R. bellii	RML An4	1.54	31.6	1311		LAOI00000000
R. bellii	RML Mogi	1.62	31.5	1336		LAOJ00000000
R. buchneri	ISO-7	1.66	32.6	1324		JFKF00000000
R. canadensis	McKiel	1.16	31.1	902		NC_009879
R. canadensis	CA410	1.15	31.1	1016		NC_016929
R. conorii	Malish 7	1.27	32.4	1227		NC_003103
R. conorii	ISTT CDC1	1.25	32.5	1200		AJVP01000000
R. conorii	A-167	1.26	33	1210		AJUR01000000
R. conorii	ITTR	1.25	32.4	1157		AJHC01000000
R. felis	URRWXCal2	1.49	32.5	1444	pRF, pRFδ	NC_007109

R. felis	LSU	1.54	32.8	1970	pRF	JSEM00000000
R. felis	LSU-Lb	1.58	33.1	1691	pRF, pLbaR	JSEL00000000
"*R. gravesii*"[a]	BWI-1	1.37	32.2	1152	pRgr	AWXL01000000
R. heilongjiangensis	054	1.28	32.3	1140		NC_015866
R. helvetica	C9P9	1.37	32.2	1114	pRhe	CM001467
R. honei	RB	1.27	32.4	1171		AJTT00000000
R. hoogstraalii	Croatica	1.48	32.4	1250		CCXM00000000
R. japonica	YM	1.28	32.4	1142		NC_016050
R. massiliae	Mtu5	1.37	32.5	1152	pRma	CP000683
R. massiliae	AZT80	1.28	32.5	1207	pRmaB	CP003319-20
R. monacensis	IrR/Munich	1.35	32.4	1447	pRM	NZ_LN794217
R. montanensis	OSU 85-930	1.28	32.6	1125		NC_017043
R. parkeri	Portsmouth	1.3	32.4	1228		NC_017044
R. parkeri	Grand Bay	1.31	32.4	1223		LAOK00000000
R. parkeri	AT#24	1.3	32.4	1226		LAOL00000000
R. parkeri	Tate's Hell	1.3	32.4	1227		LAOO00000000
R. peacockii	Rustic	1.29	32.6	927	pRpe	CP001227
"R. philipii"	364D	1.29	32.5	1218		CP003308
R. prowazekii	Madrid E	1.11	29	834		NC_000963
R. prowazekii	Rp22	1.11	29	864		CP001584
R. prowazekii	RpGvF24	1.11	29	834		CP003396
R. prowazekii	GvV257	1.11	29	829		CP003395
R. prowazekii	Dachau	1.11	29	839		CP003394
R. prowazekii	BuV67-CWPP	1.11	29	843		CP003393
R. prowazekii	Chernikova	1.11	29	845		CP003391

(continued)

Table 20.1 (continued)

Species	Strain	Genome size (Mb)	G+C content (%)	Protein-coding genes	Presence of plasmid	Accession number
R. prowazekii	Katsinyian	1.11	29	844		CP003392
R. prowazekii	Cairo	1.11	29	842		APMO00000000
R. prowazekii	Breinl	1.11	29	842		CP004889
R. prowazekii	GvF12	1.11	28.9	973		APMN00000000
R. raoultii	Khabarovsk	1.34	32.8	1334	pRa1, pRa2, pRa3, pRa4	CP010969
R. rhipicephali	3-7-female6-CWPP	1.31	32.4	1117	pRrh	NC_017042
R. rhipicephali	HJ#5	1.45	32.3	1200	pHJ51, pHJ52	CP013133
R. rhipicephali	Ect	1.27	32.6	1067		LAOC00000000
R. rickettsii	Sheila Smith	1.26	32.5	1345		NC_009882
R. rickettsii	Iowa	1.27	32.4	1384		NC_010263
R. rickettsii	Morgan	1.27	32.4	1343		CP006010
R. rickettsii	R	1.26	32.4	1334		CP006009
R. rickettsii	Arizona	1.27	32.4	1343		CP003307
R. rickettsii	Brazil	1.25	32.4	1339		CP0033075
R. rickettsii	Hauke	1.27	32.4	1347		CP003318
R. rickettsii	Colombia	1.27	32.4	1342		CP003306
R. rickettsii	Hlp#2	1.27	32.4	1339		CP003311
R. rickettsii	Hino	1.27	32.4	1346		CP0033079
R. sibirica	246	1.25	32.5	1227		AABW01000001
R. sibirica	BJ-90	1.25	32.5	1217		AHIZ00000000
R. sibirica	HA-91	1.25	32.5	1175		AHZB00000000

R. slovaca	13-B	1.27	32.5	1260		NC_016639
R. slovaca	D-CWPP	1.27	32.5	1261		CP002428
R. tamurae	AT-1	1.44	32.4	1200	Plasmid 1, Plasmid 2	CCMG01000000
R. typhi	Wilmington	1.11	28.9	817		NC_006142
R. typhi	TH1527	1.11	28.9	819		NC_017066
R. typhi	B9991CWPP	1.11	28.9	819		NC_017062
Rickettsia endosymbiont of *Ixodes scapularis*		2.02	31	2309	pReis1, pReis2, pReis3, pReis4	NZ_CM000770
Rickettsia endosymbiont of *Ixodes pacificus*	Humboldt	1.56	32.2	1294		LAOP00000000

[a]Species with as yet no standing in nomenclature are written with quotation marks

The high degree of synteny enabled the identification of an ongoing gene degradation in both genomes, that of *R. prowazekii* being at a more advanced stage of reduction than that of *R. conorii* (Ogata et al. 2001). Such a genomic degradation was found to be a common feature of intracellular bacteria, undergoing a convergent evolution (Merhej et al. 2013).

Following these pioneering works, the genomes from most of the 27 *Rickettsia* species in the nomenclature (http://www.bacterio.net/rickettsia.html), and those of several as yet taxonomically classified isolated were sequenced (Table 20.1). The analysis of the 1.1 Mb genome sequence from *R. typhi*, the agent of murine typhus, enabled the identification of a hotspot for genomic rearrangements close to the replication terminus (McLeod et al. 2004). In contrast, the genome from *R. felis*, causing flea spotted fever, in addition to its large size (1.48 Mb), exhibited a polymorphic conjugative plasmid, pRF, the first plasmid detected in a *Rickettsia* (Ogata et al. 2005; Fournier et al. 2008). In addition to pRF, a second plasmid, pLbaR, was found in a booklouse-associated *R. felis* strain (Gillespie et al. 2015a). To date, plasmids have been identified in 11 *Rickettsia* species (El Karkouri et al. 2016). In addition, this genome differed from other rickettsial genomes by being less syntenic (Fig. 20.2) than those of other species and by having a larger number of transposases, genes involved in adaptation to environment (toxin–antitoxin genes, *spoT* genes), and genes involved in adaptation to eukaryotic hosts (ankyrin- and tetratricopeptide repeat-containing genes). The analysis of the *R. felis* genome was also an occasion to confirm experimentally several phenotypic traits that were predicted *in silico*, including two types of pili, notably conjugative pili, a haemolytic activity, a

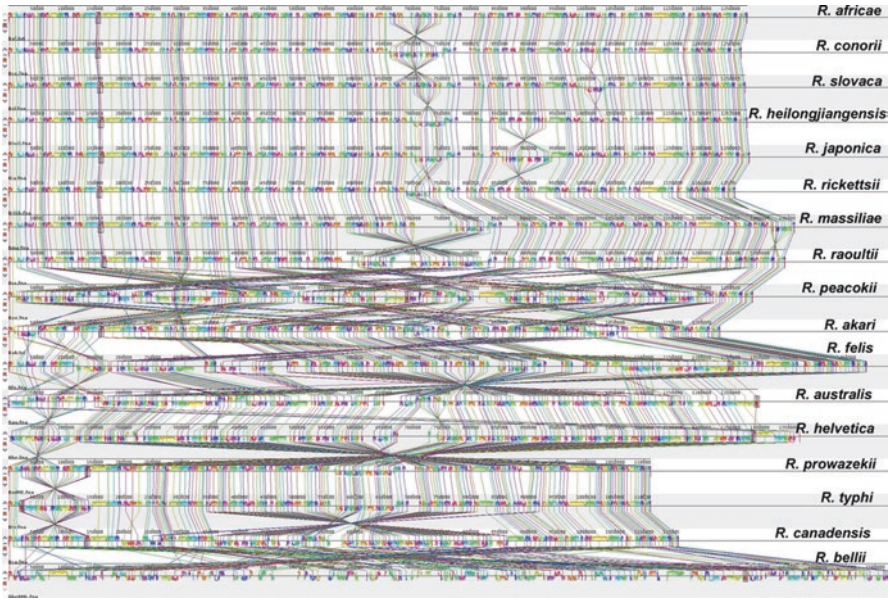

Fig. 20.2 Genomic alignment showing the high degree of synteny of most *Rickettsia* species

β-lactamase activity and intracellular motility (Ogata et al. 2005). By comparing the genomes from flea- and booklouse-borne *R. felis* strains, Gillespie et al. observed a substantial intraspecies divergence that may result from spatial isolation and host specialization (Gillespie et al. 2015a). Similar findings were reported by Bishop-Lilly et al. who described distinct genomic contents between human and squirrel *R. prowazekii* strains (Bishop-Lilly et al. 2013).

The genome of *R. bellii*, a species that has diverged early from other *Rickettsia* species but whose pathogenicity is as yet unknown, was bigger than that of *R. felis*, with 1.55 Mb, and exhibited little colinearity with other genomes (Fig. 20.2) (Ogata et al. 2006). The main finding in this genome was the identification of several features that suggested an ancestral association of rickettsiae and amoebae. In particular, *R. bellii* possessed a complete *tra* gene cluster comprised of putative conjugal DNA transfer genes, and many other genes mostly similar to homologues found in amoebal symbionts, in particular *Protochlamydia amoebophila* (Ogata et al. 2006). In addition, post-genomic analyses confirmed the presence of sex pili-like structures at the cell surface, an efficient intranuclear multiplication of *R. bellii* in eukaryotic cells, and survival in the phagocytic amoeba, *Acanthamoeba polyphaga* (Ogata et al. 2006). These findings even suggested that the ancestral association of rickettsiae and amoebae may have led to their adaptation to intracellular life within eukaryotic cells (Ogata et al. 2006).

To date, the largest rickettsial genome is that of the *Rickettsia* symbiont of *Ixodes scapularis* (REIS, 2.02 Mb, 2309 predicted ORFs) (Gillespie et al. 2012). In addition to its bigger size, the REIS genome is unique among *Rickettsia* species in that: (1) ~1/3 of its sequence is made of markers of mobile genetic elements (MGEs) and plasmids; (2) RPEs are highly divergent from those of other species; (3) 29 % of predicted ORFs do not have homologues in other rickettsiae and (4) numerous recombination events. Gene recombinations have also been identified in 79 genes of *R. felis* (Merhej et al. 2011). Wu et al. demonstrated that recombination events are not rare in *Rickettsia* genomes (Wu et al. 2009). Twenty-eight per cent of core genes exhibit evidences of recombination. This phenomenon, which occurred across all gene functional categories, especially those involved in cell wall/membrane/envelope biogenesis (Wu et al. 2009), may play an important role in the evolution of these bacteria, notably by enabling them to adapt to specific hosts.

All of the other *Rickettsia* genomes available to date, except that of *R. peacockii*, exhibited a high degree of synteny with those of *R. conorii*, *R. massiliae*, *R. prowazekii* and *R. typhi* (Fig. 20.2). Overall, rickettsial genomes are undergoing a progressive degradation process, range in size from 1.1 to 2.1 Mb and exhibit a G+C content of 29–33 %. By analysing 43 rickettsial genomes, Duan et al. calculated that their pangenome was made of 4837 genes (Duan et al. 2014). The almost perfect colinearity of most *Rickettsia* genomes (Fig. 20.1), enabled to explore the mechanism of their reduction, based on a gradual gene degradation. Several successive steps were identified in this degradation process, from intact ORFs to transcribed split ORFs (presence of stop codons), to split ORFs no longer transcribed, to still recognizable ORFs missing start and/or stop codon(s), to complete gene disappearance. However, rickettsial genomes undergo a complex evolution in which reduction is

partially balanced with duplications of several genetic elements. The *R. conorii* genome not only contained numerous RPE copies, but also duplications of genes encoding: (1) ADP/ATP translocases enabling exploitation of ATP produced by the host cell, and possibly involved in adaptation to starvation; (2) proline/betaine transporters enabling transfer of osmolytes, notably proline, in the cytoplasm and presumably linked to reactivation (Renesto et al. 2005); (3) toxin–antitoxin modules known to be involved in programmed cell death. Five toxin–antitoxin modules are present in most SFG *Rickettsia* genomes (Georgiades and Raoult 2011); (4) SpoT, an hydrolase involved in the metabolism of guanosine tetra- (ppGpp) and pentaphosphates (pppGpp) and overexpressed in situations of starvation. Five *spoT* genes are found in *Rickettsia* genomes (Renesto et al. 2005) and (5) surface cell antigens (SCA) responsible for antigenic differences among *Rickettsia* species. Seventeen paralogous genes encoding SCA membrane proteins were identified in *Rickettsia* species (Blanc et al. 2005). These surface-exposed autotransporter proteins play roles in adhesion to host cells. Evolutionary analysis demonstrated that they were undergoing a dual selection pressure: the auto-transporter domain has evolved under purifying selection whereas the passenger domain has evolved under positive selection (Blanc et al. 2005).

3 Mobile Genetic Elements and Horizontal Gene Transfer

Rickettsia species were initially thought to be devoid of mobile genetic elements until the discovery of the pRF plasmid in *R. felis* in 2005 (Ogata et al. 2005). pRF was the first putative conjugative plasmid identified in an obligate intracellular bacterium and, intriguingly, existed in a short and a long form (Fournier et al. 2008). To date, plasmids have been identified in at least 11 *Rickettsia* species (Table 20.1) (Fournier et al. 2008). A characteristic of rickettsial plasmids (RPs) is that they vary in number and type depending on the strain, even in a given species (Baldridge et al. 2008; Fournier et al. 2008; El Karkouri et al. 2016; Rolain et al. 2009). As a consequence, the absence of plasmids in the genomes from some species does not obligately imply that they are plasmidless. This was demonstrated in *R. africae*, *R. akari*, *R. bellii* and *R. felis* in which the plasmid content is strain dependent (Fournier et al. 2008, 2009; Baldridge et al. 2008). In addition, we have observed that plasmids may be lost through passage in cell culture, as in *R. felis* (Fournier et al. 2008). RPs range in number and size per species from 1 to 4 plasmids and from 12 kb to 83 kb, respectively, and contain 15–85 genes. A total of 260 distinct orthologous genes have been identified in RPs (Gillespie et al. 2007). Several of these rickettsial plasmid genes have homologues in their resident chromosomes, suggesting that ORFs from rickettsial plasmids were incorporated into the chromosomes (Gillespie et al. 2007). In a recent study investigating the evolutionary history of 20 RPs belonging to 11 species (El Karkouri et al. 2016), we inferred that the last common plasmid ancestor ("pRICO") of the current RPs had been vertically inherited mainly from *Rickettsia/Orientia* chromosomes and diverged vertically into a single or multiple plasmid(s) in

each species. RPs harbour genes encoding for DNA replication (*dnaA*) and partitioning (*parA*), heat shock proteins, conjugation (*tra* clusters) and mobilization (transposons, integrases). RPs appear to have undergone a complex evolution, including horizontal gene transfer (HGT) events, gene duplication and genesis, as well as gene degradations and reductive evolution similar to that observed in rickettsial chromosomes, possibly leading to cryptic plasmids or complete plasmid loss. RPs exchanged genes mainly with species belonging to α/γ-proteobacteria lineages including rickettsial and non-rickettsial genomes. Therefore, RPs are mosaic structures that may play biological roles similar to or distinct from their co-residing chromosomes. The RP genes involved in nucleotide/carbohydrate transport and metabolism were under the influence of vertical evolution only, whereas those involved in cell wall/membrane/envelope biogenesis, cycle control, amino acid/lipid/coenzyme and secondary metabolites biosynthesis, transport and metabolism underwent mainly horizontal transfer events (El Karkouri et al. 2016).

In addition to plasmids, other mobile genetic elements (MGEs) have been identified in rickettsial genomes. The genome from REIS is composed of 35 % of MGEs including transposases, integrases, phage-related ORFs, four plasmids and a nine copy-*Rickettsiales* amplified genetic element (RAGE) encoding a F-type type IV secretion system (T4SS) (Gillespie et al. 2012). RAGE modules had previously been found in *Orientia tsutsugamushi*, the agent of scrub typhus and a member of the order *Rickettsiales*, and are also present in the pLbaR plasmid of *R. felis* strain LSU-Lb (Gillespie et al. 2015a), in *R. bellii* and *R. massiliae* (Blanc et al. 2007; Ogata et al. 2006). In REIS, RAGE modules are sufficiently divergent from each other to have resulted from multiplied genomic invasions (Gillespie et al. 2012). RAGE modules are facilitators of gene exchange (Gillespie et al. 2015b).

In addition, the genome from *R. peacockii* exhibits 42 chromosomal copies of the ISRpe1 transposon (Felsheim et al. 2009). These transposons were likely acquired by HGT from a *Cardinium* species and caused deletions. Finally, the pRpe plasmid from *R. peacockii* carries a partial glycosylation island acquired from *Pseudomonas aeruginosa* (Felsheim et al. 2009).

Initially thought to have a limited impact on rickettsial genomes due to the intracellular life of these bacteria, HGT is now recognized as an important evolutionary force for these bacteria. In addition to the above-described examples, rickettsial genomes exhibit several other evidences of HGT, including plasmid- or chromosome-encoded conjugation genes (*tra* cluster), *pat*2, T4SS and ATP/ADP translocases (Merhej and Raoult 2011). These genes were likely acquired by a rickettsial ancestor from other bacteria living in amoebae and then vertically inherited (Merhej and Raoult 2011). However, Weinert et al. demonstrated that conjugation genes, notably the *tra* cluster, were common in the genus *Rickettsia* and were frequently horizontally transferred between strains, in contrast with the rest of rickettsial genes (Weinert et al. 2009). They also proposed that the *tra* cluster in *R. massiliae* resulted from a recent transfer event (Weinert et al. 2009). In contrast, in *R. felis*, a partial *tra* cluster was found encoded on a plasmid (Ogata et al. 2006). The role of conjugation genes in *Rickettsia* species is uncertain as they are absent in many of the vertebrate pathogenic species (Weinert et al. 2009). However, HGT-acquired genes are heterogenously

distributed among rickettsial genomes, both in terms of presence/absence and, when present, in terms of numbers (Blanc et al. 2007). Murray et al. observed that genomes with more conjugation genes were larger (Murray et al. 2016). However, these authors also suggested that the phylogenetic incongruences between gene and core genome phylogenies should be interpreted cautiously as they may result from errors in phylogenetic tree reconstruction and thus are not systematically markers of HGT (Murray et al. 2016).

4 Do Genomes Shed Light on Rickettsial Pathogenesis?

Among the 17 pathogenic *Rickettsia* species, the degree of virulence varies greatly, from *R. prowazekii*, the agent of epidemic typhus, a deadly disease exhibiting a 30 % mortality (without treatment) to *R. africae*, the agent of the mild ATBF, with no reported fatality. In addition, intraspecific variations in virulence have been noted, notably for *R. rickettsii* (Paddock et al. 2014), *R. prowazekii* (Bechah et al. 2010) and *R. conorii* (Fournier and Raoult 2009). In 2007, Darby et al., studying the available genomes from members of the order *Rickettsiales*, highlighted the absence of an association between pathogenicity and the acquisition of novel virulence genes (Darby et al. 2007). In a genomic comparison of species causing rickettsioses of various severities, we also observed that *R. prowazekii*, the most virulent species, had the smallest genome and had no additional gene when compared to milder species (Fournier et al. 2009). We speculated that genome degradation, notably through the loss of regulatory genes, caused an increased virulence of *Rickettsia* species not only in humans but also in their arthropod hosts (Fournier et al. 2009). This phenomenon has also been observed in other bacterial genera (Merhej et al. 2013).

However, several other factors may also be involved in the observed differences in pathogenicity among *Rickettsia* species. In 2008, Ellison et al. compared the genomes of a virulent and an avirulent *R. rickettsii* strains (Ellison et al. 2008). The authors identified 143 deletions and 492 single nucleotide polymorphisms (SNPs) between the two strains. Among these genetic events, a deletion in the rOmpA-coding gene and a defective processing of the rOmpB protein, two major antigens of *Rickettsia* species, were proposed to explain the difference in pathogenicity (Ellison et al. 2008). rOmpA and rOmpB proteins are members of the SCA family. These proteins play roles in adhesion to, and invasion of eukaryotic host cells, and evasion from host complement-mediated clearance (Li and Walker 1998; Cardwell and Martinez 2009; Chan et al. 2009; Riley et al. 2012). Clark et al. confirmed the previous findings, only detecting, in addition to the precited rOmpA and rOmpB defects, a limited number of SNPs and insertion/deletion events between the genomes from two virulent and two avirulent *R. rickettsii* strains (Clark et al. 2015). Felsheim et al. also identified a mutated *ompA* gene and a deletion of *sca1* in *R. peacockii* that may explain the absence of pathogenicity of this species (Felsheim et al. 2009).

By comparing the genomes from virulent and avirulent *R. prowazekii* strains, we observed hotspots of mutations in poly(A) and poly(T) in eight genes in the avirulent strain. These included *recO* that encodes for a protein playing a role in DNA reparation and whose mutation might result in loss of virulence (Bechah et al. 2010), The study of the genome from *R. heilongjiangensis*, a Chinese pathogenic species enabled the prediction of virulence factors including toxins, cell surface proteins and T4SS components (Duan et al. 2014). Using genomic comparison, Gillespie et al. demonstrated that all *Rickettsia* species have a chromosomally encoded atypical P-type T4SS that lacks the *virB5* gene (Gillespie et al. 2009). In addition, its components are scattered in five distinct fragments of the genomes (Gillespie et al. 2009). In *Rickettsia* species, the P-type T4SS appears to play a role in protein secretion (Gillespie et al. 2015b). In addition, Duan et al. demonstrated that, among T4SS genes, *virB2* might be essential for virulence in *R. prowazekii* as strain MadridE lacked this gene that was present in all pathogenic strains of this species (Duan et al. 2014). Some *Rickettsia* species also possess an F-type T4SS that is either encoded on the chromosome and/or a plasmid and is part of the RAGE module identified in REIS (nine copies), *R. bellii* and *R. massiliae* (one copy each) (Gillespie et al. 2015b). *Rickettsia* genomes possess genes encoding for the Adr1 and Adr2 adhesins that also enable evasion from host complement-mediated clearance (Riley et al. 2014). In addition, the *rickA* gene encodes for a protein involved in host cell actin polymerization (Gouin et al. 2004). However, the role of this protein in cell-to-cell spread is uncertain, as *R. canadensis* has a complete *rickA* gene but is not able to polymerize actin (Balraj et al. 2008). All *Rickettsia* genomes also possess the *tlyA* and *tlyC* genes that encode putative haemolysins, the latter of which was demonstrated to exhibit a membranolytic activity in eukaryotic cells (Radulovic et al. 1999). Furthermore, two genes encoding patatin-like proteins with phospholipase activity are present in all genomes and may play a role in phagosomal escape (Gillespie et al. 2015b). Ankyrin- and tetratricopeptide repeat-containing proteins have been demonstrated to play a role in cell infection in *R. typhi* (Kaur et al. 2012). These repeated elements are present in a greater number of copies in larger *Rickettsia* genomes (Merhej and Raoult 2011). Finally, the toxin–antitoxin (TA) modules found in most SFG rickettsiae may also play a role in rickettsial pathogenesis, in particular as a control mechanism in case of starvation (Georgiades and Raoult 2011). VapC, one of these toxins, was notably demonstrated to have an RNAse activity in the host cell (Audoly et al. 2011). However, the exact role pf TA modules is unclear as they are not found in TG rickettsiae but present in multiple copies in some SFG species that are less pathogenic (Georgiades and Raoult 2011).

In the *R. prowazekii–R. conorii* genome comparison, we had observed that some of the intergenic spacers were highly conserved (Ogata et al. 2001; Merhej and Raoult 2011). In the conserved spacers from 13 *Rickettsia* species, Schroeder et al. identified 1785 small non-coding RNAs (sRNAs) (Schroeder et al. 2015). These ubiquitous genetic elements have been demonstrated in enterobacteriaceae to play important roles in virulence (Lee and Groisman 2010). In an experimental model of human endothelial cell infection, these authors further confirmed the expression of six of these sRNAs in *R. prowazekii*, thus suggesting that they might be involved in

virulence regulation and host adaptation in this species (Schroeder et al. 2015). However, Matelska et al. observed that rickettsial genomes, like those of *Buchnera* and *Mycoplasma* species, had less sRNAs than free-living bacteria (Matelska et al. 2016), possibly due to a loss of non-essential transcriptional regulators following adaption to intracellular life (Fuxelius et al. 2008).

Therefore, the pathogenicity of *Rickettsia* species is likely to result from a combination of mechanisms but these intrications remain unknown.

5 Conclusions

Due to improved diagnostic methods and increased interest, the number of representatives of the genus *Rickettsia* has increased dramatically over the past 20 years, with 27 currently validated species and several dozens of as-yet unclassified isolates or genotypes. These strictly intracellular bacteria have undergone a specific genomic evolution marked by a progressive gene degradation resulting from their adaptation to eukaryotic host cells. Despite their small genomes, some of these bacteria have retained a conjugation apparatus and duplicates of various genetic elements such as RPEs, ADP/ATP translocases, proline/betaine transporters, toxin–antitoxin modules, SpoT and SCA-encoding genes. Combined with proteomic analyses, comparative genomics has enabled the identification of various host cell invasion and virulence mechanisms. However, further studies are mandatory to fully understand the pathogenicity differences among *Rickettsia* species.

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Part IX
Wolbachia

Chapter 21
Wolbachia

Benjamin L. Makepeace and A. Christina Gill

1 Host Range and Distribution

In contrast to the other members of the *Anaplasmataceae* such as *Anaplasma*, *Ehrlichia, Neorickettsia, Neoehrlichia,* and *Aegyptianella*, *Wolbachia* is apparently incapable of infecting vertebrates. However, due to the lifestyle of certain invertebrate hosts of *Wolbachia*, which are themselves parasites of vertebrates, *Wolbachia* can come into contact with the mammalian immune system and acquire some of the characteristics of a bacterial pathogen. Moreover, *Wolbachia* has an intimate relationship with many arthropod vectors of vertebrate pathogens, such that it may have an indirect, but nevertheless potentially very important, role to play in disease transmission.

Wolbachia pipientis was formally described from the mosquito *Culex pipiens* by Marshall Hertig (Hertig 1936). However, it was not until the advent of PCR and pioneering work involving the screening of a wide diversity of arthropods that the true scale of the distribution of *Wolbachia* began to be understood (Werren et al. 1995). It is now estimated that 52 % of terrestrial arthropod species are infected with *Wolbachia*, which implies that its total catalogue of hosts is in the range of several millions (Weinert et al. 2015). Therefore, if mitochondria are discounted, *Wolbachia* is probably the most successful vertically transmitted symbiont on the planet and has been described as driving a "pandemic" among arthropods (Bordenstein et al. 2006). Indeed, PCR screening has identified *Wolbachia* infections in almost all of the orders within the subphylum Hexapoda (Table 21.1), with the Phasmatodea (stick insects or walking sticks) representing the only case where a reasonably extensive survey failed to detect infected species (Perez-Ruiz et al. 2015). *Wolbachia* are also widespread in the class Arachnida (subphylum Chelicerata; Table 21.1), and a recent analysis suggests that the incidence of this symbiont in hexapods and

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Table 21.1 Taxonomic classification of *Wolbachia* hosts

Phylum	Subphylum	Class	Order	Common name
Arthropoda	Hexapoda	Insecta	Archaeognatha	Jumping bristletails
			Blattodea	Cockroaches and termites
			Coleoptera	Beetles
			Diptera	True flies
			Hemiptera	True bugs
			Hymenoptera	Wasps, bees, and ants
			Lepidoptera	Moths and butterflies
			Mantodea	Mantises
			Neuroptera	Net–winged insects
			Odonata	Dragonflies and damselflies
			Orthoptera	Grasshoppers and crickets
			Phthiraptera	Lice
			Plecoptera	Stoneflies
			Psocoptera	Booklice
			Siphonaptera	Fleas
			Strepsiptera	Twisted–wing parasites
			Thysanoptera	Thrips
		Entognatha	Collembola	Springtails
	Crustacea	Maxillopoda	Pedunculata	Goose barnacles
		Ostracoda	Podocopida	Seed shrimp
		Malacostraca	Isopoda	Woodlice, pill bugs, and sea slaters
			Amphipoda	Scuds and sandhoppers
	Chelicerata	Arachnida	Trombidiformes	Chiggers and spider mites
			Sarcoptiformes	Moss mites and mange mites
			Parasitiformes	Ticks and predatory mites
			Araneae	Spiders
			Pseudoscorpionida	Pseudoscorpions
			Scorpiones	Scorpions
Nematoda	–	Chromadoria	Rhabditida	Roundworms

chelicerates is not significantly different (Weinert et al. 2015). However, in certain groups such as the mites, *Wolbachia* is apparently found in fewer superfamilies than another abundant obligate intracellular symbiont, *Cardinium* (phylum Bacteroidetes) (Chaisiri et al. 2015). Of note, all of the major groups of dipteran vectors (Culicidae, Phlebotominae, Simuliidae, and Ceratopogonidae) contain species infected with

Wolbachia, as do the Phthiraptera (lice), Siphonaptera (fleas), and ectoparasitic bugs in the families Reduviidae and Cimicidae (Table 21.1). *Wolbachia* DNA sequences have also been detected in PCR and 16S rRNA amplicon sequencing surveys of several tick species, although a report of *Wolbachia* infection in a major European tick species, *Ixodes ricinus*, was found to originate from a parasitoid wasp and not the tick itself (Tijsse–Klasen et al. 2011).

The picture in the third major arthropod subphylum, the Crustacea, is less clear (Table 21.1). *Wolbachia* has long been recognised as widespread in terrestrial isopods such as woodlice, which is consistent with the hypothesis that the *Wolbachia* pandemic is essentially a terrestrial phenomenon. Caution in interpretation of the current data is warranted, however, as screening of *Wolbachia* in marine organisms appears to have been limited to date. While the reported presence of *Wolbachia* from isopods and amphipods from the marine littoral zone could represent a secondary invasion from terrestrial habitats (Cordaux et al. 2001), the recent detection of *Wolbachia* in a fully marine species, the goose barnacle *Lepas anatifera* (Cordaux et al. 2012), suggests that the true reach of the pandemic may have been underestimated.

Although electron microscopic studies of parasitic nematodes in the family Onchocercidae (the filarial nematodes or filariae) had revealed bacterial symbionts as early as the mid–1970s (Kozek and Marroquin 1977), it was not until 1995 that molecular techniques identified *Wolbachia* in this group (Sironi et al. 1995). The impact of this discovery on the *Wolbachia* field was considerable, since filariae are responsible for two of the world's most important neglected tropical diseases: lymphatic filariasis or "elephantiasis" (44 million people infected and 2 million years lived with disability) and onchocerciasis or "river blindness" (17 million people infected and 1.2 million years lived with disability) (Naghavi et al. 2015). Furthermore, *Wolbachia* is also a symbiont of a major parasite of dogs and cats, the heartworm *Dirofilaria immitis*. Initially, the presence of *Wolbachia* in the filarial nematodes was assumed to be a consequence of their close relationship with arthropods, as they are transmitted by blood–feeding vectors (such as mosquitoes) that often harbour their own *Wolbachia* symbionts. However, PCR screens of other nematode families that parasitise arthropods (Mermithidae, Steinernematidae, Heterorhabditidae, and Dracunculidae) have failed to identify *Wolbachia* infections (Duron and Gavotte 2007; Foster et al. 2014). To date, the only non–filariid nematode family in which an extant *Wolbachia* symbiosis has been identified is the Pratylenchidae, which has been reported to contain a single infected genus, *Radopholus* (Haegeman et al. 2009). Remarkably, this is a burrowing nematode of plants that lacks an arthropod vector. Thus, while nematodes have not been subjected to PCR screening for *Wolbachia* on the same scale as that for arthropods, the general absence of *Wolbachia* sequences in the rapidly expanding genomic and transcriptomic datasets for non–filariid nematodes suggests that it is extremely uncommon within the phylum as a whole. Even within the Onchocercidae, surveys of *Wolbachia* in parasites of wild vertebrates indicate that <40 % of species are infected, and it is very rare or completely absent in the ancestral subfamilies of the filariae (Ferri et al. 2011).

The Arthropoda and the Nematoda are the two phyla incorporating the vast majority of species in the superphylum Ecdysozoa, the group of animals that undergoes ecdysis or moulting of the cuticle. Indeed, it has been speculated that the success of *Wolbachia* might be attributable to a puppet–master–like manipulation of ecdysteroid hormones. However, screening for *Wolbachia* in minor ecdysozoan phyla such as the Tardigrada (water bears), Nematomorpha (horsehair worms), and Priapulida (penis worms) has been very limited or non–existent to date.

2 Transmission

Although a number of factors combine to drive the unparalleled distribution of *Wolbachia* in arthropods, highly efficient vertical transmission is undoubtedly the most important and this has been estimated as a rate of 97 % in wild *Drosophila melanogaster* (Hoffmann et al. 1998). In this species, *Wolbachia* becomes localised in the germline precursor cells or "pole cells" by concentrating in the posterior of mature oocytes, where the pole cells develop during embryogenesis. The targeted migration of *Wolbachia* towards the posterior pole is achieved via transportation along microtubules by the motor protein, kinesin–1 (Serbus and Sullivan 2007). Surprisingly, this direct infection of the germline precursor cells to ensure transmission to the oocytes of the next generation is not ubiquitous within the *Drosophila* genus. In a series of elegant experiments, it was demonstrated that a more prevalent strategy (used in addition to germplasm infection by *D. melanogaster*, but as the sole mechanism by several other *Drosophila* spp.) of ensuring vertical transmission is colonisation of the somatic stem–cell niche in the germarium and invasion of oocytes during oogenesis (Frydman et al. 2006; Toomey et al. 2013). In contrast, transmission via the germline stem–cell niche is a less commonly used route (Fast et al. 2011; Toomey et al. 2013). *Wolbachia* are also present in the male germline of *Drosophila*, although they become excluded from sperm cysts during the latter stages of spermatogenesis and hence cannot be transmitted paternally. Targeting of the stem–cell niche of the *Drosophila* testis is less prevalent than for the ovary, and this so–called "hub tropism" in male flies is controlled by both *Wolbachia*– and host–derived factors (Toomey and Frydman 2014), whereas in females, *Wolbachia*–derived factors directing stem–cell tropism predominate (Toomey et al. 2013). Similar studies in filarial nematodes have highlighted some interesting parallels with vertical transmission in *Drosophila*. In nematode oocytes, *Wolbachia* also aggregates towards the posterior pole and segregates asymmetrically during embryogenesis in cells destined to become the somatic hypodermal cords and the germline precursors (Landmann et al. 2010). However, the symbionts are subsequently lost from the germline precursors and must reinvade the female reproductive tract by a tortuous route at the young adult stage, crossing from the hypodermal cords (Fig. 21.1) to the ovarian stem–cell niche (the distal tip cell) and proliferating in the rachis before cellularisation of the oogoniae (Landmann et al. 2012; Fischer et al. 2011).

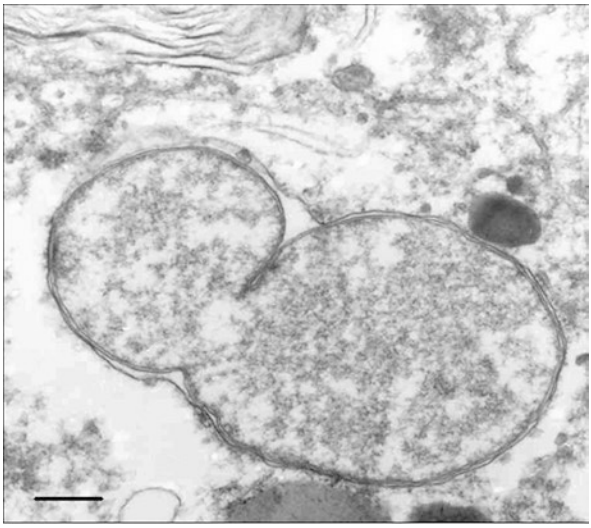

Fig. 21.1 Location of *Wolbachia* in filarial nematodes. A dividing *Wolbachia* cell (surrounded by a host–derived vacuolar membrane) within the hypodermal cord syncytium of an adult filarial worm specimen (*Onchocerca ochengi*). In filarial nematodes of medical or veterinary importance, *Wolbachia* is located in the somatic hypodermal cords of both sexes and in the female reproductive tract. To infect the germline, *Wolbachia* must traverse the somatic tissues in young adult worms to reach the ovarian distal tip cell. Scale bar, 0.5 μm

These indirect methods of infecting the female germline appear to reflect an ancestral ability of *Wolbachia* to establish vertical transmission in an opportunistic manner after horizontal transmission events, which are probably relatively rare in nature. Thus, following experimental infection of *D. melanogaster*, *Wolbachia* located outside the reproductive tract are able to cross three barriers (the peritoneal sheath membrane, the muscle epithelium surrounding the ovariole, and the somatic tissues enclosing the germline) to reach the developing oocytes (Frydman et al. 2006). Since in most arthropod groups, *Wolbachia* phylogenies are not congruent with those of their hosts, horizontal transmission is clearly a significant evolutionary force contributing to the spread of the symbionts. A number of different routes for horizontal transmission have been proposed, with varying degrees of laboratory– and field–based evidence available for each. Insect parasites and parasitoids of other arthropods may mechanically transmit *Wolbachia* between hosts on their mouthparts or ovipositor, as has been reported for the wasp *Eretmocerus* sp. nr. *furuhashii* parasitising whiteflies (Ahmed et al. 2015). Scavenging, predation, and cannibalism are also likely to be major routes, with experimental evidence supporting these modes of transmission between terrestrial isopods (Le Clec'h et al. 2013) and also between *D. melanogaster* and the mould mite, *Tyrophagus putrescentiae* (Brown and Lloyd 2015). Furthermore, arthropod communities co–feeding on the same material might transfer *Wolbachia* via plant or fungal tissue, which has been suspected following analysis of *Wolbachia* gene sequences from specialised insect

communities exploiting habitats such as mushrooms (Stahlhut et al. 2010), fig syconia (Shoemaker et al. 2002; Yang et al. 2012), and pumpkins (Sintupachee et al. 2006). Finally, the presence of *Wolbachia* in the guts of the sterile worker castes of ants, coupled with evidence of excretion of organisms in faeces, suggests that at least in some cases, *Wolbachia* may have adapted to use horizontal transmission as a significant mode of dissemination even on ecological timescales (Frost et al. 2014).

3 Taxonomy

The taxonomic classification of *Wolbachia* remains controversial. Discounting "*Wolbachia persica*" (now reclassified as *Francisella persica* in the γ–*Proteobacteria*) (Larson et al. 2016) and "*Wolbachia melophagi*", which is probably a species of Bartonella (order *Rhizobiales*) (Maggi et al. 2009), W. pipientis is the only formally recognised species within the genus. However, the wide host range of *Wolbachia* and apparently fundamental differences in the natural history of this symbiont between arthropod and nematode hosts have led to a status quo in which the genus name is commonly used without the specific epithet. *Wolbachia* strains were divided into phylogenetic "supergroups" originally using single–gene analyses (based on the *Wolbachia* surface protein gene, wsp; the 16S rRNA gene, or ftsZ) (Zhou et al. 1998; O'Neill et al. 1992). Subsequently, evidence of recombination in wsp led to the adoption of a multilocus sequence typing system based on five other genes (Baldo et al. 2006), with *wsp* classification reserved for a supplemental level of fine–scale strain resolution. Gradually, the simplistic model in which arthropods were infected with supergroup A and B strains, which had undergone promiscuous horizontal transfer; whereas filarial nematodes were infected with supergroup C and D strains, which strictly co–evolved with their hosts (Bandi et al. 1998), has given way to a new universe of expanding *Wolbachia* diversity encompassing at least 16 supergroups. Of these, C, D, and J are apparently restricted to filarial nematodes, F is the only known supergroup found both in filarial nematodes and arthropods, and the taxonomic status of the strain identified in the burrowing nematode *Radopholus similis* remains controversial (Koutsovoulos et al. 2014; Haegeman et al. 2009). All other supergroups are restricted to arthropods, and some such as E and H are confined to specific taxa (springtails and termites, respectively). Recent analyses using 90 orthologous gene clusters applied to partial or complete *Wolbachia* genomes from the major supergroups (A–F and H) lend strong support to these clades as robust phylogenomic entities (Gerth et al. 2014).

As the phylogeny of *Wolbachia* became recognised as increasingly complex, a proposal to formally consolidate all of the supergroups into the single species *W. pipientis* was put forward (Lo et al. 2007). This was not accepted by the *Wolbachia* research community as a whole, and was especially controversial among those working on *Wolbachia* in filarial nematodes, in which the biology of the symbionts seemed quite distinct from that of the "typical" strains infecting arthropods (Pfarr et al. 2007). Furthermore, genomic analyses of supergroup A and B *Wolbachia*

co–infecting a single host, *Drosophila simulans*, revealed a high degree of genetic isolation, supporting a species–level classification (Ellegaard et al. 2013). In 2015, a toolkit consisting of average nucleotide identity, in silico DNA–DNA hybridisation, GC content and synteny analyses was applied to current *Wolbachia* genome data and led to a proposal to formally describe seven new *Wolbachia* species, with *W. pipientis* reserved for supergroup B only (Ramirez–Puebla et al. 2015). Interestingly, in this scheme, individual supergroups were sometimes designated to contain more than one species. Whether this new classification will come to be widely accepted by the *Wolbachia* research community remains to be seen.

4 Reproductive Manipulations

Wolbachia is famous for its association with four reproductive phenotypes in arthropod hosts: male killing, feminisation, induction of parthenogenesis, and cytoplasmic incompatibility (Werren et al. 2008). Although various other selfish genetic elements and microbes are capable of orchestrating one or more of these manipulations, *Wolbachia* is the only known organism that can encompass all four strategies to enhance its vertical transmission (Fig. 21.2). In male killing, reported from the Coleoptera, Lepidoptera, Pseudoscorpiones, and Diptera, elimination of males during early embryonic development enhances female fitness by reducing sibling competition and (in some species) by providing food in the form of unhatched eggs containing dead male embryos. Feminisation, the conversion of genetic males into functional females, is most widespread in the Malacostraca, but has also been reported from certain Hemiptera and Lepidoptera. Induction of female parthenogenesis (thelytoky) has been described from the Hymenoptera, Thysanoptera, Collembola, and Trombidiformes and enables females to develop from unfertilised eggs which, in haplodiploid species, would develop into males in the absence of infection (arrhenotoky). All three of these mechanisms cause sex ratio distortion in favour of females and thus promote vertical transmission at the expense of males, which are dead–end hosts. The final form of reproductive manipulation, cytoplasmic incompatibility (CI), is the most widespread strategy deployed by *Wolbachia* and is more subtle than the other phenotypes in that sex ratio biases are not induced (except in some haplodiploid species). This phenotype has been identified in a diverse array of hosts across the Hexapoda, Chelicerata, and Crustacea. In unidirectional CI, a fitness benefit for infected females is established by rendering matings between infected males and uninfected females infertile, whereas all other possible combinations result in viable offspring (Fig. 21.2). However, complicated patterns of bidirectional CI can also occur in some species groups, such as the *C. pipiens* complex. Here, crosses between infected males and infected females are only fertile if the *Wolbachia* strains they carry are compatible with one another.

Although CI is the most intensely studied of the reproductive manipulations induced by *Wolbachia*, its molecular basis has remained elusive. For the past 20 years, analyses of CI have progressed within a framework called the “modification–rescue

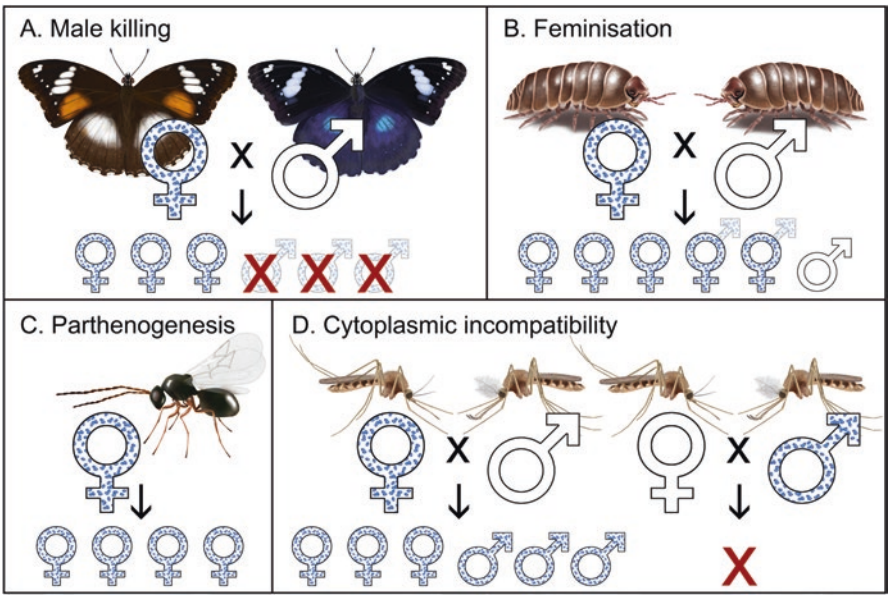

Fig. 21.2 Examples of the major types of reproductive manipulations associated with the maternally transmitted endosymbiont, *Wolbachia*. (**a**) In the blue moon butterfly *Hypolimnas bolina*, *Wolbachia* infection results in the death of male embryos, producing all–female broods; (**b**) In the pill bug *Armadillidium vulgare*, infection with *Wolbachia* converts genetic males into functional females resulting in female–biased sex ratios; (**c**) In the parasitic wasp *Leptopilina clavipes*, *Wolbachia* infection is associated with gamete duplication allowing parthenogenesis, the asexual production of offspring from unfertilised ova; (**d**) In males of the common house mosquito *Culex pipiens*, *Wolbachia* infection causes alterations that lead to developmental arrest of growing embryos, a phenotype which is rescued if the female in the pair is also infected with a compatible *Wolbachia* strain

model", in which a modification factor or toxin imprinted onto sperm from infected males interferes with the normal programme of embryogenesis if the fertilised egg lacks *Wolbachia* (Mercot and Poinsot 2009). Conversely, in infected eggs, the rescue factor or antitoxin provides an antidote to the modification factor and restores compatibility, leading to successful embryonic development. The modification and rescue factors are hypothesised to be different genetic determinants, sometimes referred to as the "lock" and the "key", respectively, which directly interact with one another. However, the patterns of bidirectional CI observed in the *C. pipiens* complex among other arthropod groups indicate that several pairs of lock and key genes must exist, leading to populations of *Wolbachia* variants that are incapable of rescuing CI induced by a "foreign" modification factor (Atyame et al. 2014).

Although the modification and rescue factors underpinning CI have yet to be conclusively identified, the mechanics of CI at the cytological level have been described in detail, and the potential list of candidate *Wolbachia* proteins involved in CI is gradually becoming shorter. Universally across host taxa, CI causes

asynchronous mitotic divisions of the male and female pronuclei during early embryogenesis. Hence, deposition of the histone H3.3/H4 complex, completion of chromosome replication, and activation of the kinase Cdk1 are delayed in the male pronucleus relative to its female counterpart, leading to activation of S–phase checkpoints and a lag in male nuclear envelope breakdown (Landmann et al. 2009). Consequently, at metaphase, the paternal chromosomes are not fully condensed and at anaphase, they do not properly segregate, becoming stretched at the centrosome poles or excluded from the embryo entirely. In some haplodiploid species, this leads to the development of viable male progeny from CI crosses (Tram et al. 2006). Whatever the molecular nature of the rescue factor in infected embryos may be, it is clear that it restores synchrony in the cell cycle between male and female pronuclei.

Comparative genomics of incompatible CI–causing *Wolbachia* strains, particularly those infecting mosquitoes of the *C. pipiens* group, have identified several candidate effector proteins associated with prophage regions. Some of these proteins contain ankyrin–repeat domains, which have long been known to mediate protein–protein interactions in eukaryotic cells (Siozios et al. 2013). In certain lines of *Culex quinquefasciatus*, two ankyrin–encoding genes displayed non–synonymous differences between incompatible *Wolbachia* strains, and one of these genes was expressed in a sex–specific manner in the mosquito host (Sinkins et al. 2005). Additional circumstantial evidence implicating ankyrin–repeat proteins in the mediation of CI was obtained by genomic comparisons between *w*Mel (a CI–inducing *Wolbachia* strain from *D. melanogaster*) and *w*Au (a non–CI–inducing strain from *D. simulans*), which revealed significant sequence differences in ankyrin genes between these strains (Sutton et al. 2014). However, transgenic expression of nine candidate ankyrin protein genes in *D. melanogaster* failed to induce or modify CI, suggesting poor expression in the host (for instance, due to codon usage), degradation of the protein products, a requirement for accessory *Wolbachia* proteins, or simply selection of inappropriate candidates (Yamada et al. 2011). A similar approach was taken following analysis of genomic differences between incompatible *Wolbachia* strains derived from *C. pipiens* f. *molestus* and *C. quinquefasciatus*, which identified three genes in inserted or deleted regions from the former (Pinto et al. 2013). One of these was not an ankyrin–repeat coding gene, but a transcriptional regulator called *wtrM*. Intriguingly, when *wtrM* was expressed in transfected *C. quinquefasciatus* mosquitoes, the insects responded by upregulation of a homolog of a *D. melanogaster* gene called grauzone, a regulator of female meiosis. Unfortunately, as *wtrM* failed to express in ovaries, the CI phenotype could not be tested in the transgenic mosquitoes. Finally, an alternative approach to candidate discovery of CI is proteomic analysis of *Wolbachia*–modified sperm collected from the spermathecae of female mosquitoes. When applied to sperm of *C. pipiens* f. *pipiens*, a single uncharacterised protein, WPIP0282, was identified, which has orthologs only in *Wolbachia* strains that are capable of inducing CI (notably, it is absent from the genome of *w*Au) (Beckmann and Fallon 2013; Sutton et al. 2014).

The reproductive manipulations orchestrated by *Wolbachia* can be powerful evolutionary forces in infected arthropod populations, and can lead to rapid symbiont invasions across vast geographic scales. For instance, the rate of spread of the CI–

inducing strain *w*Ri in *D. simulans* across both California and eastern Australia has been estimated at 100 km per year (Kriesner et al. 2013; Turelli and Hoffmann 1991). *Wolbachia* invasions can also lead to fascinating arms races with their hosts, such as the case of the male–killing strain *w*Bol1 in the butterfly, *Hypolimnas bolina* (Fig. 21.2). Using DNA analysis of museum specimens collected from islands of the Indian and Pacific Oceans, dramatic swings in sex ratio and *Wolbachia* prevalence in this species have been identified over the past 120 years, resulting from initial spread of the male–killer followed by the evolution of effective countermeasures in some populations (Hornett et al. 2009). The *H. bolina* system also teaches another important lesson about *Wolbachia*, in that male butterflies carrying a suppressor allele may survive to adulthood, but their infection still manifests as CI, revealing an underlying plasticity to the *Wolbachia* phenotype (Hornett et al. 2008). However, mathematical models demonstrate that reproductive manipulations are, at best, only half of the secret behind *Wolbachia*'s success and cannot account for the pandemic on their own (Fenton et al. 2011). Indeed, the majority of *Wolbachia* infections in arthropod hosts have no known phenotype.

5 Obligate Dependencies

Following the discovery of *Wolbachia* in filarial nematodes, two key observations were made that appeared to separate these symbionts from their congeners in arthropods. First, symbiosis was a fixed trait for infected filarial species; second, the phylogeny of the symbionts and their hosts showed strong evidence of co–cladogenesis (Bandi et al. 1998; Taylor and Hoerauf 1999). These are hallmarks of an obligate mutualistic relationship and accordingly, tetracycline treatment of *Wolbachia*–infected filarial nematodes in rodent models led to prophylactic effects, growth retardation, and sterilisation of adult female worms (McCall et al. 1999; Bandi et al. 1999; Hoerauf et al. 1999), whereas naturally aposymbiotic (symbiont–negative) filariae were unaffected (Hoerauf et al. 1999). Further studies in a natural host–parasite system, *Onchocerca ochengi* in cattle, demonstrated that adult worms could be killed by prolonged oxytetracycline treatments (Fig. 21.3) (Langworthy et al. 2000). However, the physiological basis of the *Wolbachia*–filaria dependency has remained elusive.

Genome sequencing of strain *w*Bm (supergroup D) from *Brugia malayi*, a parasite of humans causing lymphatic filariasis, revealed complete pathways for the biosynthesis of riboflavin and heme, which cannot be produced *de novo* by *B. malayi* itself (Foster et al. 2005). This suggested a scenario akin to the provisioning of amino acids or B–vitamins by the γ–proteobacterial symbionts *Buchnera* and *Wigglesworthia* in pea aphids and tsetse flies, respectively. In both of these cases, the symbionts effectively supplement nutrients that are lacking in the diet of their host insect. However, there were problems in applying this model to the filarial nematodes. First, heme auxotrophy is apparently universal in the Nematoda, while symbiosis in either free–living or parasitic species appears to be relatively rare

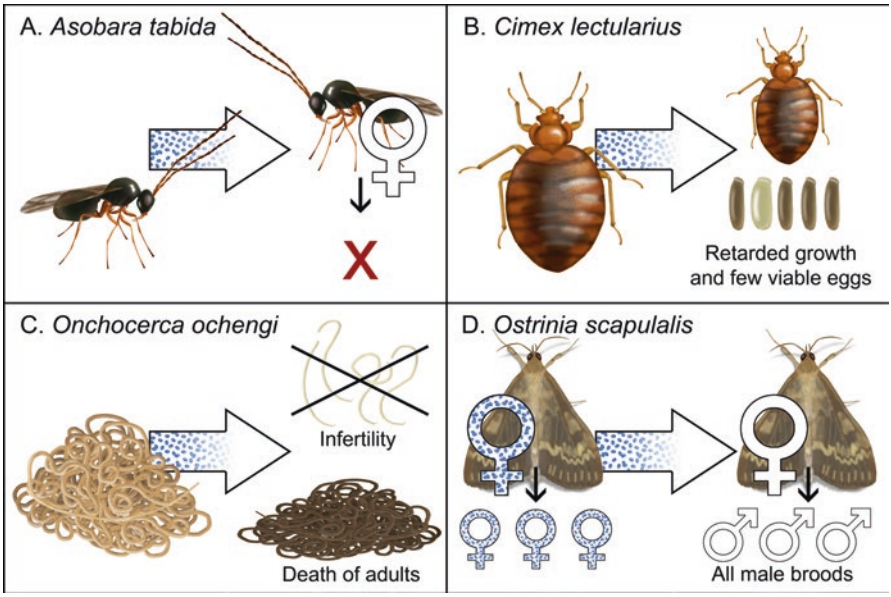

Fig. 21.3 Examples of obligate *Wolbachia*–host associations. Large arrows indicate removal of *Wolbachia* by antibiotic treatment. (**a**) In the parasitic wasp *Asobara tabida*, elimination of a particular strain of the symbiont leads to catastrophic nurse cell apoptosis in the ovaries leading to failure of oogenesis and infertility; (**b**) Removal of *Wolbachia* in the bedbug *Cimex lectularius* results in a large reduction of the number of eggs that develop normally, a prolonged nymphal period, and a lower adult emergence rate. These effects can be reversed by supplementing the bedbug's diet with B–vitamins, indicating that *Wolbachia* normally provides these essential nutrients to the host; (**c**) In the filarial nematode *Onchocerca ochengi*—a parasite of cattle and the closest known relative of the human parasite *Onchocerca volvulus*—elimination of the bacterial symbionts leads to infertility and eventually results in the death of adults; (**d**) In the adzuki bean borer *Ostrinia scapulalis*, the presence of *Wolbachia* results in lethal feminisation of males resulting in all–female broods. When *Wolbachia* are removed, the situation is reversed and genetic females die, producing all–male broods

across the phylum, suggesting that nematodes routinely obtain heme from their diet. Indeed, blood–feeding has been reported in filarial nematodes (George et al. 1985; Attout et al. 2005), and most filariae do not harbour *Wolbachia* or any other symbionts. Second, the heme pathway is required for the production of various enzymes in bacteria and is rarely lost, except in some highly specialised pathogens that scavenge heme from the host (Smalley et al. 2011). On the other hand, experimental evidence for riboflavin provisioning in *B. malayi* has been obtained in vitro, in that extraneous riboflavin can partially rescue the reduction in motility and microfilaria (first–stage larva) production in adult female worms exposed to doxycycline (Li and Carlow 2012).

The picture was complicated further by sequencing of the first *Wolbachia* genome from supergroup C, which was obtained from *O. ochengi* (the closest relative of the human "river blindness" parasite, *Onchocerca volvulus*) (Darby et al. 2012).

Strain *w*Oo has retained the heme pathway, but the capability for riboflavin biosynthesis has been lost via pseudogenisation. Other pathways for cofactors and vitamins are poorly presented in the *w*Oo genome, and expression of the heme biosynthesis genes in adult worms was low, although transcripts involved in ATP production were well represented. Subsequently, experiments in *B. malayi* demonstrated protein–protein interactions in the vacuoles surrounding the symbionts between host glycolytic enzymes and proteins derived from the *Wolbachia* surface (Melnikow et al. 2013). This suggests that both supergroup C and D symbionts perhaps have a supplementary mitochondrion–like role in filarial nematodes.

Within a few years of the discovery of the mutual dependency between *Wolbachia* and filarial nematodes, a case of an obligate *Wolbachia*–arthropod relationship was uncovered. In the parasitic wasp *Asobara tabida*, removal of *Wolbachia* strain *w*Atab3 by antibiotic treatments results in the inability of females to produce mature oocytes (Fig. 21.3) (Dedeine et al. 2001). The mechanistic basis of this dependency centres on control of apoptosis, which becomes dysregulated following removal of the symbiont, leading to collapse of nurse cell function in the egg chambers (Pannebakker et al. 2007). The characterisation of the *A. tabida* system was a seminal milestone in *Wolbachia* research, as it highlighted an underappreciated aspect of symbiosis: evolved dependencies are not necessarily mutualistic in nature [extensively reviewed by Zug and Hammerstein (2015)]. However, among arthropods, the *Wolbachia* symbiosis in *A. tabida* remains unusual as an example of obligate dependency without an underlying reproductive phenotype.

In the moth *Ostrinia scapulalis*, *Wolbachia* is an obligate reproductive parasite, since it is required for female development. Although infected females of *O. scapulalis* produce all–female broods, *Wolbachia* is not a typical male–killer in this system, because removal of *Wolbachia* during the larval stage leads to all–male broods in the next generation due to death of genetic females (Fig. 21.3) (Kageyama and Traut 2004). This is a fascinating example of *Wolbachia* hijacking the sex determination system of a lepidopteran, in which females are generally the heterogametic sex (ZW). Normal phenotypic sexual development in lepidopterans requires the expression of sex–specific isoforms of the doublesex gene (*dsx*) and in *O. scapulalis*, removal of *Wolbachia* results in the expression of the male–specific isoform irrespective of the genetic sex. This discordance between phenotypic and genetic sex is lethal, and suggests that *Wolbachia* has functionally replaced the feminising factor (*Fem*) on the W chromosome that operates upstream of *dsx*. Indeed, recent experiments in the related species *Ostrinia furnacalis* have demonstrated that *Wolbachia* infection in genetic males markedly reduces transcript levels of the masculinising gene *Masc* located on the Z chromosomes, which is normally suppressed by Fem in genetic females (Fukui et al. 2015). This leads to failure of dosage compensation and embryonic lethality that can be rescued by injection of artificial *Masc* transcripts.

Lepidopterans are not the only arthropod group in which reproductive manipulations have led to obligate dependencies on *Wolbachia*. Haplodiploids can be particularly susceptible to reproductive "takeovers" as they are prone to evolve to asexuality, a phenomenon addressed by the "functional virginity" hypothesis (Zug and

Hammerstein 2015). For instance, in female–biased *Asobara japonica* populations from the main islands of Japan, which are invariably infected with *Wolbachia*, removal of the symbiont does not result in failure of oogenesis (as in *A. tabida*), but rather in all–male broods (Kremer et al. 2009a). Thus, *Wolbachia* induces thelytokous parthenogenesis in *A. japonica*, as is the case in many other haplodiploid species. However, while these male wasps remain sexually functional, females no longer attract males; therefore, *Wolbachia* has become indispensable for the production of daughters. Remarkably, uninfected *A. japonica* populations from the southern islands of Japan exhibit normal sex ratios and retain an arrhenotokous mode of reproduction, in which unfertilised eggs develop into males. This suggests that the spread of *Wolbachia* and the evolution of dependence in the more northerly populations of *A. japonica* is a relatively recent phenomenon, driven by selection on female wasps to restore a more balanced sex ratio. In arrhenotokous species, this can only be achieved by reducing the rate of fertilisation, ultimately producing "functional virgins" that can no longer reproduce sexually and require *Wolbachia* to propagate.

Perhaps the most dramatic case of *Wolbachia* affecting sexual behaviour occurs in the neotropical *Drosophila paulistorum* complex. In this system of six sympatric semispecies, *Wolbachia* infection induces bidirectional CI in hybrid crosses, with >90 % of embryos failing to develop normally (Miller et al. 2010). Furthermore, the few male flies that reach maturity exhibit *Wolbachia* over–replication in the testes and are rendered sterile. Unexpectedly, antibiotic curing of *Wolbachia* infections in *D. paulistorum* is invariably lethal, whereas mild antibiotic treatments that reduce *Wolbachia* density have detrimental phenotypes including reduced female fecundity and male–biased progeny. However, the most striking phenotype of symbiont depletion is reduced discrimination in mate choice between incompatible semispecies, revealing that *Wolbachia* controls premating isolation and thus drives speciation in this complex.

These examples demonstrate that most obligate dependencies on *Wolbachia* in arthropods can hardly be described as mutualistic in the strong sense, as the host clearly does not benefit from the hijacking of its mode of reproduction. In contrast, the *Wolbachia* symbiosis in bedbugs displays all of the hallmarks of a classical mutualism. As for tsetse flies and aphids, the symbionts in bedbugs are located in a specialised organ, the bacteriome. Removal of *Wolbachia* from bedbugs using antibiotic treatments induces a developmental phenotype (reduced embryonic survival and nymphal growth; Fig. 21.3), but in contrast to the examples above, this could be rescued by supplementation with B–vitamins (Hosokawa et al. 2010). Typically, obligate hematophagous arthropods such as tsetse flies rely on symbionts for B–vitamin supplementation due to the low levels of these nutrients in blood, and sequencing of the supergroup F *Wolbachia* (*w*Cle) genome from bedbugs revealed complete pathways for both riboflavin and biotin (Nikoh et al. 2014). Interestingly, the biotin biosynthetic cluster in this genome showed evidence of acquisition by lateral gene transfer from another endosymbiont, and was also present in the genome of a filarial *Wolbachia* strain (*w*Oo), albeit in a pseudogenised (inactivated) form. Further experiments to dissect the contribution of individual B–vitamins to the rescue of the *Wolbachia* depletion phenotype showed that provisioning of riboflavin

plus biotin restores adult emergence rate and fecundity to normal levels. Although the symbiosis between *w*Cle and bedbugs remains the only thoroughly characterised case of a genuine *Wolbachia* mutualism in arthropods, other such symbioses no doubt exist. An intriguing example involving plant parasitism occurs in the leaf miner *Phyllonorycter blancardella* (Lepidoptera), in which the photosynthetic patches or "green islands" on the senescent leaves of apple trees (occupied by the moth larvae in the autumn) are lost if *Wolbachia* is removed (Kaiser et al. 2010). This implicates *Wolbachia* in the control of plant cytokinin levels that maintain these green islands.

6 Host–Symbiont Interactions

As mentioned above, *Wolbachia* has become essential in *A. tabida* due to uncontrolled apoptosis in nurse cells following symbiont removal, which results in failure of oogenesis. This phenotype reflects an intimate integration of *Wolbachia* into the physiology of its host, which is most extreme in obligate dependencies, but extends as a continuum into facultative symbioses. Thus, in *Drosophila mauritiana*, *Wolbachia* is not essential, but infected females exhibit a clear reproductive advantage over uninfected individuals, producing on average four times more eggs (Fast et al. 2011). In parallel with the *A. tabida* system, this effect on oogenesis is due to inhibition of apoptosis by *Wolbachia*, although it occurs at a later stage (in the germarium) rather than in the pre–vitellogenic stages. More extensive apoptosis after antibiotic treatment occurs in the obligate symbiosis between *w*Bm and *B. malayi*, affecting developing embryos and subsequent larval stages, which accounts for the sterilising and prophylactic activity of tetracyclines against filarial nematodes (Landmann et al. 2011). Interestingly, apoptosis in embryos and microfilariae was not restricted to *Wolbachia*–infected cells (which are few in number in these stages), whereas the somatic hypodermal cords, which contain a high density of *Wolbachia*, did not undergo apoptosis. These "non–cell–autonomous" effects suggest that *Wolbachia*, primarily located in the hypodermal cords, secretes molecules which inhibit apoptosis in the developing stages in utero. Importantly, the lack of apoptosis observed in the hypodermal cords indicates that the mechanism by which adult nematodes are killed following antibiotic treatment is distinct from that affecting the embryos and larval stages.

A second fundamental cellular process that is inhibited by *Wolbachia* is autophagy, the process by which cells control the recycling of superfluous or dysfunctional organelles and cytoplasmic material by degradation in lysosomes. In *B. malayi*, markers of autophagy co–localised with *Wolbachia*–rich areas in nematode stages that contain the highest density of symbionts (L4 and adult females) (Voronin et al. 2012). Remarkably, when various *B. malayi* stages were exposed to an activator of autophagy (rapamycin), *Wolbachia* loads were decreased by a similar level to that achieved using doxycycline. Tissue sections from rapamycin–treated nematodes displayed increased rates of autophagosome–lysosome fusion accompanying the

destruction of *Wolbachia*. Conversely, inhibition of an initiator of autophagy (ATG1) by RNA interference led to a significant increase in symbiont density in adult females. Stimulating autophagy using rapamycin or RNAi targeting an inhibitor of autophagy also decreased *Wolbachia* load in mosquito and *Drosophila* cell lines, and in infected *D. melanogaster* flies. The mechanism by which *Wolbachia* suppresses autophagy is not known, but may involve regulation of the autophagy activator ATG8a in the vacuole surrounding the symbionts.

Not all *Wolbachia* infections inhibit autophagy. In terrestrial isopods, artificial transfer of strain *w*VulC from the natural host, *Armadillidium vulgare*, to an experimental host, *Porcellio dilatatus dilatatus*, causes deleterious effects including a reduction in growth and motility, changes in behaviour, and a shortening of lifespan (Le Clec'h et al. 2012). These were accompanied by profound changes to the central nervous system of the foreign host, most notably autophagic vesicles and vacuoles within the adipocytes and nerve cells. Importantly, the symbiont density in these tissues was not significantly higher than that observed with *w*VulC in its natural host, indicating that it was the excessive autophagic response of *P. d. dilatatus* rather than *Wolbachia* over–replication *per se* that was responsible for the pathogenic phenotype.

Although much research has focused on potential host benefits in (putatively) mutualistic *Wolbachia* symbioses, recent experiments have sought to address the balance by identifying the resources that may be consumed by *Wolbachia*. Genomic analyses have confirmed that *Wolbachia* lacks the capacity for *de novo* amino acid synthesis (with the exception of *meso*–diaminopimelate in most strains), and prediction of metabolic networks suggests that amino acid catabolism is its prime source of energy (Foster et al. 2005; Wu et al. 2004; Darby et al. 2012). In Aedes aegypti mosquitoes infected with strain *w*MelPop and fed animal sources of blood, which have lower levels of amino acids than the preferred human blood, a profound decrease in fertility and egg viability is observed (Caragata et al. 2014). Such effects can be partially rescued by supplementation of sheep blood with amino acids, revealing a potential competitive interaction between female mosquitoes and *Wolbachia* for these nutrients under limiting conditions. Although this particular host–symbiont relationship is an artificial one, it is certainly plausible that such competition occurs in natural systems. The situation for riboflavin is less clear. While this represents the sole B–vitamin that has a universally conserved biosynthetic pathway in *Wolbachia* (except in strain *w*Oo), suggesting that it could be provisioned to the host, experimental evidence for this has only been obtained in bedbugs (Moriyama et al. 2015). In *Aedes albopictus* cells transfected with strain *w*Str, an inhibitor of riboflavin uptake enhanced growth of host cells and depleted *Wolbachia*, whereas uninfected mosquito cells exhibited suppressed growth in the presence of this inhibitor (Fallon et al. 2014). The most likely interpretation of these data, which should be treated with caution considering the artificial nature of the system, is that *Wolbachia* reserves riboflavin production for its own needs and when starved of riboflavin, infected host cells obtain a benefit from reduced symbiont growth, leading to less competition for other resources.

A more global approach to the effects of diet on life–history traits between infected and uninfected hosts has been undertaken recently. By modifying the ratio of yeast (protein) and sucrose (carbohydrate) in diets fed to *D. melanogaster*, it was found that egg production in infected flies is limited by protein availability (Ponton et al. 2015). Conversely, when carbohydrate is limiting, survivorship of infected flies was significantly reduced relative to their uninfected counterparts. Interestingly, when flies were offered a food choice between yeast and sucrose to optimise the protein:carbohydrate ratio, infected flies consumed relatively more carbohydrate, suggesting that longevity was prioritised over reproductive rate. In a separate study using *D. melanogaster*, yeast–enriched diets were demonstrated to decrease *Wolbachia* loads in the oocytes while having the opposite effect in somatic tissues, whereas sucrose–enriched diets increased *Wolbachia* density in oocytes but showed no effect on somatic symbiont loads (Serbus et al. 2015). The impact of high dietary yeast intake on germline *Wolbachia* density were not driven by the amino acid content of the food, but by insulin–mediated stimulation of the nutrient–responsive kinase complex, TORC1, via insulin–producing cells in the brain. Furthermore, the increase in *Wolbachia* load observed in oocytes following maintenance on a high–sucrose diet was also dependent on insulin signalling. Changes in *Wolbachia* cell morphology in oocytes when dietary intake of yeast was high suggested that the bacteria were undergoing a stress response, although the precise mechanism remains unclear.

One micronutrient, iron, has had a key role to play in our developing conception of *Wolbachia*–host interactions even if a detailed understanding of its role in the symbiosis remains frustratingly incomplete (Gill et al. 2014). A potential role for iron was brought to the fore when the hypothesis that heme is provisioned by *Wolbachia* in filarial nematodes was first posited (Foster et al. 2005). However, it was rapidly realised that the flux of iron in general between *Wolbachia* and its host might have profound effects on the nature of the symbiosis. Indeed, although *Wolbachia* confers a fecundity benefit on *D. melanogaster* females fed on ecologically relevant low–iron diets, this is also the case if flies are fed diets with artificially elevated levels of iron, which are likely to increase oxidative stress in the absence of detoxification (Brownlie et al. 2009). These data indicate that the mechanism underpinning the fecundity benefits involves iron buffering rather than simple provisioning, even if *Drosophila* are unlikely to encounter high dietary iron in nature. Further support for a central role for iron metabolism in *Wolbachia* symbioses was provided by the *A. tabida* system, in which removal of *Wolbachia* upregulates a host iron–storage protein, ferritin (Kremer et al. 2009b). When *A. tabida* parasitised *D. melanogaster* larvae fed on a high–iron diet, wasp emergence was significantly decreased, but comparisons between *Wolbachia*–infected and aposymbiotic wasps demonstrated that *Wolbachia* partially mitigated the effect. Female wasps emerging under high–iron conditions displayed increased levels of apoptosis in their ovaries even when *Wolbachia* was present, revealing an exquisite sensitivity to iron–mediated oxidative stress in this system. In the facultative symbiosis between strain *w*Ri and *D. simulans*, infected flies absorbed more iron when the levels available in their food was increased, which was associated with increased expression of

Wolbachia bacterioferritin but not host ferritin. A similar compensatory response of bacterioferritin, in the absence of an increase in host ferritin expression, was also observed in a mosquito cell line artificially infected with *Wolbachia* and exposed to elevated iron in the medium (Kremer et al. 2009b).

Whether variation in dietary iron is of importance for *Wolbachia* symbiosis in the natural environment is very challenging to test directly. However, control of iron metabolism by *A. tabida* does appear to be central to the evolution of dependence on *Wolbachia* in the wild, as variation in the severity of the ovarian phenotype (no egg production versus some egg production with aborted larval development) following symbiont removal is correlated with the degree of ferritin expression in different *A. tabida* populations across France (Kremer et al. 2010). That is, severe phenotypes (no eggs) in aposymbiotic females are linked with ferritin upregulation, whereas milder phenotypes (some eggs) are not. This same pattern was observed for expression of transferrin and superoxide dismutase, highlighting the expected association between iron metabolism and the control of oxidative stress.

7 Interactions with Co–infecting Organisms

In 2008, two seminal studies on the impact of *Wolbachia* on viral infections in insects were published that totally revolutionised the field. Both studies reported that *Wolbachia* infections in *D. melanogaster* increased fly survival following challenge with RNA viruses, either by inhibiting viral accumulation or by enhancing the tolerance of the flies to viral infection (Hedges et al. 2008; Teixeira et al. 2008). In one study, the effect of *Wolbachia* on the progression of a DNA virus infection was also tested, but here no protection was apparent (Teixeira et al. 2008). The *Wolbachia* strains used in these experiments included *w*MelPop, a life–shortening, pathogenic variant that evolved in a laboratory stock of *D. melanogaster* and which does not exist in nature. Prior to the viral protection studies, intensive efforts were underway to transfect *Ae. aegypti*, which naturally lacks *Wolbachia*, with derivatives of *w*MelPop in an effort to shorten the lifespan of this major dengue vector in the wild (Rasgon et al. 2003). The new data revealing the viral protection phenotypes in *Drosophila* spurred a race to determine if *w*MelPop could reduce the ability of *Ae. aegypti* (and perhaps other mosquitoes) to transmit viruses, over and above the potential to shorten lifespan.

The presence of *w*MelPop in *Ae. aegypti* was found to block or dramatically reduce the dissemination and replication of dengue virus in mosquito tissues and the production of viral particles in saliva (Moreira et al. 2009). *Wolbachia* infection also successfully suppressed the dissemination of chikungunya virus in *Ae. aegypti*, and even reduced the prevalence and load of oocysts of Plasmodium gallinaceum, a species of avian malaria. This latter finding extended the pathogen interference phenotype of *w*MelPop to a non–viral pathogen (albeit a species that does not affect humans). Remarkably, *w*MelPop was subsequently shown to inhibit the life cycle of a filarial nematode, *Brugia pahangi*, in *Ae. aegypti* and to confer protection against

a Gram–negative bacterium, *Pectobacterium carotovorum* (Kambris et al. 2009). The race was then on to determine if *Wolbachia* would reduce the development of human malaria parasites, especially *Plasmodium falciparum* (the most deadly form), in *Anopheles* spp. mosquitoes. These experiments present substantial technical challenges, as *Anopheles* spp. are generally refractory to *Wolbachia* infection. Initially, transient somatic infections with *w*MelPop or *w*AlbB (from Ae. albopictus) were shown to significantly suppress *P. falciparum* oocyst numbers in *Anopheles gambiae*, the primary African malaria vector (Kambris et al. 2010). When a technical breakthrough was finally achieved with *Anopheles stephensi* (the main malaria vector from South Asia), which established vertically transmitted germline infections of strain *w*AlbB, substantial reductions in *P.* falciparum oocyst and sporozoite densities were observed in transfected mosquitoes (Bian et al. 2013).

These groundbreaking findings present something of a paradox: if *Wolbachia* can block pathogen transmission in major vector species under laboratory conditions, how is it that many competent vectors are infected with *Wolbachia* in nature? For instance, in contrast with *Ae. aegypti*, *Ae. albopictus* is naturally superinfected with two *Wolbachia* strains, *w*AlbB and *w*AlbA, and remains a significant vector of arboviruses in many locations worldwide. Intriguingly, these natural *Wolbachia* infections were associated with more homogeneous loads of chikungunya virus in *Ae. albopictus* from La Réunion island relative to aposymbiotic mosquitoes, although *Wolbachia* density decreased as the viral infection progressed (Mousson et al. 2010). In contrast, using the same strain of *Ae. albopictus, Wolbachia* infection was associated with a reduced density of dengue virus in the salivary glands and an absence of infectious viral particles in saliva, even though viral replication in other mosquito organs was not inhibited overall (Mousson et al. 2012). Subsequently, studies of *Wolbachia*–pathogen interactions in other natural systems have highlighted some significant areas of concern, including instances of potentially increased vector competence. Thus, in the case of *C. quinquefasciatus* naturally infected with *w*Pip, the presence of *Wolbachia* increases the susceptibility of mosquitoes to infection with an avian malaria parasite, *P. relictum*, which is transmitted by *C. quinquefasciatus* in the wild (Zele et al. 2014). Moreover, wPip extends the longevity of *C. quinquefasciatus* and reduces mosquito mortality associated with *P. relictum* infection, parameters that are likely to be critical for overall vectorial capacity (Zele et al. 2012).

These contrary outcomes in different *Wolbachia*–vector–pathogen systems emphasise the importance of understanding the mechanistic basis of pathogen interference (or enhancement) in order to make predictions about the likely impact of manipulating *Wolbachia* infections for disease control (Fig. 21.4). Initial studies on *Ae. aegypti* transfected with *w*MelPop showed a potential role for the upregulation of immune effector genes encoding antimicrobial peptides in pathogen interference (Moreira et al. 2009). Furthermore, genes involved in the opsonisation response were upregulated in *An. gambiae* harbouring somatic infections with *w*MelPop (Kambris et al. 2010), whereas stable transfection of *Ae. aegypti* with wAlbB led to the production of reactive oxygen species, stimulation of the Toll innate immune pathway, and release of antimicrobial peptides that could limit dengue virus

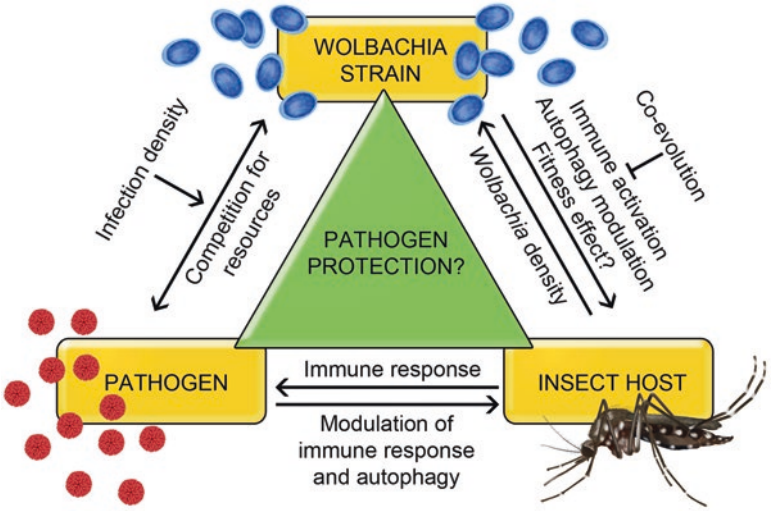

Fig. 21.4 Interactions between the insect host and *Wolbachia* that could lead to protection of the host from pathogens. In general, host–*Wolbachia* associations provide pathogen protection only when *Wolbachia* reach a high density within the host, suggesting that the bacteria may be reducing success of the pathogen by competing for resources. Additionally, *Wolbachia* infection has various effects on the host which could result in pathogen protection. These effects include activation of the immune system (in particular via the Toll pathway) and modulation of autophagy pathways. The nature of these host–*Wolbachia* interactions is thought to depend on whether or not the host species has co–evolved and hence adapted to *Wolbachia* infection, with evidence for immune activation involving mainly *Wolbachia*–naïve species. When exploiting *Wolbachia*–mediated pathogen protection for the control of pathogens of human importance, a further consideration is whether or not there are any fitness costs to the host related to the *Wolbachia* infection. A negative impact on host fitness may prevent the introduction of *Wolbachia* infection into wild mosquito populations

proliferation in midgut and fat body tissues (Pan et al. 2012). However, blockage of dengue transmission could be demonstrated in an Ae. albopictus line in which the natural *Wolbachia* superinfection was removed and replaced with strain wMel, despite only a transient increase in the expression of immune–related transcripts (Blagrove et al. 2012). Neither was pathogen interference dependent on induction of reactive oxygen species in *Ae. albopictus* transfected with *w*Mel (Molloy and Sinkins 2015). Finally, strains wMel and *w*MelPop successfully impeded dengue virus infection in *D. melanogaster* in the absence of any discernible immune–priming effect (Osborne et al. 2012), and *Wolbachia*–infected *Drosophila* mutants lacking components of the small interfering RNA pathway, a key component of innate antiviral immunity, did not show reduced resistance to *Drosophila* C virus (DCV) and Flock House virus (FHV) (Hedges et al. 2012). These data suggest that while novel *Wolbachia* infections lead to an upregulation of immune pathways, especially in hosts such as *Ae. aegypti* and *An. gambiae* which usually lack *Wolbachia* infections, this phenomenon is not necessary for significant interference to occur with pathogen establishment and dissemination (Fig. 21.4).

Systematic studies to dissect the specific factors that contribute to antiviral protection were performed in isogenic *D. simulans* lines infected with 19 different *Wolbachia* strains of *Drosophila* origin and then challenged with DCV and FHV (Martinez et al. 2014). Approximately half of the *Wolbachia* strains tested conferred protection, which was strongly correlated with the density attained by the symbionts and a corresponding reduction in viral titre. Analysis of expression of immune genes confirmed that antiviral effects were not associated with immune priming, while reduction of *Wolbachia* density using sub–curative antibiotic treatments abated viral interference. These data are in accordance with analysis of *Wolbachia* density in transfected mosquitoes, such as *Ae. aegypti* carrying *w*AlbB and *Ae. albopictus* infected with *w*Mel, in which the symbionts achieve much higher loads than are observed naturally with wAlbB and *w*AlbA in Ae. albopictus (Blagrove et al. 2012; Lu et al. 2012).

An inverse correlation between symbiont and viral densities suggests competition over intracellular resources. One key nutrient that cannot be synthesised *de novo* by either insects or members of the Anaplasmataceae is cholesterol, which is also required for cellular entry and replication of many viruses. Accordingly, in *Wolbachia*–infected *D. melanogaster* fed a cholesterol–rich diet, DCV killed flies several days earlier than was the case for flies reared on standard diet (Caragata et al. 2013). This decrease in the protective effect of *Wolbachia* was associated with elevated viral loads, but was not correlated with changes in *Wolbachia* density. Thus, competition between viruses and *Wolbachia* for cholesterol is likely to be a key determinant of the viral interference phenotype, although this does not rule out a role for other lipids and dietary components in modulating the strength of the effect (Fig. 21.4).

While the fanfare surrounding pathogen interference by *Wolbachia* has understandably focused on the potential benefits for disease control (see below), a second major implication of these findings relates to the spectacular spread of *Wolbachia* in arthropod populations. Might the *Wolbachia* pandemic be driven by the induction of defence against natural enemies rather than (or at least as well as) reproductive manipulations (Fenton et al. 2011)? This is an attractive hypothesis, but one that is supported by little evidence to date because the systems currently examined (with the exception of DCV in *Drosophila*) are usually host–*Wolbachia*–pathogen combinations that do not exist in nature. In *D. melanogaster*, the *w*MelCS–like strains that confer the greatest antiviral protection (at least under laboratory conditions) shorten host lifespan compared with wMel–like variants, which exhibit lower levels of protection (Chrostek et al. 2013). In the wild, these *w*Mel–like variants have largely replaced the *w*MelCS clade, suggesting that the costs of reduced lifespan outweigh the benefits of antiviral protection. Moreover, whereas *w*Pip provides protection against mortality caused by *P. relictum* in *C. quinquefasciatus* (Zele et al. 2012), a male–killing *Wolbachia* strain in the African armyworm *Spodoptera exempta* (Lepidoptera) significantly increases susceptibility to a natural nucleopolyhedrovirus infection (Graham et al. 2012). Clearly, more research involving naturally occurring interactions is required.

8 Genomics and Mobile Genetic Elements

Since the publication of the first *Wolbachia* genome (strain *w*Mel from supergroup A) in 2004, the total volume of available genomic data for this symbiont has exploded in parallel with the advent of next–generation sequencing, although the number of complete (i.e. circularised chromosomes) for different *Wolbachia* strains remains very modest at approximately 10. The most heavily sampled clade is supergroup A with four complete genomes and numerous partial assemblies, although at least one circularised genome each is now available for supergroups B, C, D, and F. While substantial reductions in sequencing costs and the associated expansion of access to sequencing facilities have facilitated the creation of whole genome assemblies for many *Wolbachia* hosts, both in the Arthropoda and the Nematoda, bacterial sequences are routinely removed from eukaryotic genomes during bioinformatic filtering steps. Even where conscious efforts have been made to retrieve *Wolbachia* genome data from host genome sequencing projects (or indeed, sequencing of the host genome was simply a means to obtain a *Wolbachia* genome), the depth of coverage of the *Wolbachia* data is dependent on the density of the symbionts and may be too low to allow accurate predictions of gene content. Thus, *Wolbachia* genome projects often remain dependent on laborious purification of symbionts from bulk preparations of host material (whole organisms or cell lines), although significant progress has been made on the development of probe array capture techniques to isolate *Wolbachia* sequences from the host homogenate prior to sequencing (Geniez et al. 2012; Kent et al. 2011).

Our understanding of *Wolbachia* genomics has been largely shaped by the detailed comparisons made between *w*Mel (from *D. melanogaster*) and the second genome to be sequenced, *w*Bm (from *B. malayi*). The wMel genome (~1.3 Mb) highlighted a number of features which, at that time, had not been observed previously in obligate intracellular bacterial genomes, including a high density of repeats, insertion sequences (IS; including some potentially functional copies), group II introns, and three prophage elements (Wu et al. 2004). These features were conspicuously absent in the *w*Bm genome (~1.1 Mb), although approximately 50 inactivated IS copies were apparent (Foster et al. 2005). Moreover, the *w*Mel genome encodes for 23 ankyrin–repeat domain proteins, whereas the *w*Bm genome contains only five. Thus, it was hypothesised that exposure of *w*Mel to opportunities for gene exchange via horizontal transmission events, coupled with its potential to induce CI, had shaped more of a dynamic genome compared to that of *w*Bm, which showed a more obvious signature of stability imposed by strict vertical transmission.

Sequencing of additional genomes from arthropods and nematodes has generally maintained this picture, with some important caveats. The *Wolbachia* genome of the tsetse fly *Glossina morsitans morsitans* (strain *w*Gmm) is apparently smaller than that of other arthropod *Wolbachia* genomes at ~1.0 Mb and contains only 10 ankyrin–repeat domain proteins (Brelsfoard et al. 2014). Moreover, it seems to carry very few repeats and mobile genetic elements. However, the assembly of the *w*Gmm genome is highly fragmented, suggesting that repetitive elements in

particular, which can be challenging to assemble, may be underrepresented in the current draft. Conversely, sequencing of strain *w*Ls, a supergroup D symbiont from the filarial nematode *Litomosoides sigmodontis*, revealed 210 IS copies accounting for 12 % of the genome (the greatest proportion in a *Wolbachia* genome described to date), although all of these are non–functional (Comandatore et al. 2015). The supergroup F genome of strain *w*Cle from bedbugs also provided some surprises, as it remains relatively large (~1.3 Mb) despite its mutualistic role, and also harbours a large number of IS copies and several group II introns (Nikoh et al. 2014; Comandatore et al. 2015). This may reflect a relatively recent shift to a mutualistic relationship with the host.

Unexpected differences in the structure of *Wolbachia* genomes from supergroups C and D became apparent as filarial genome projects progressed, producing several complete or otherwise highly contiguous symbiont genome assemblies. Strain *w*Oo from *O. ochengi* was the first complete supergroup C genome to become available, and was shown to be remarkably degenerated, with almost 200 pseudogenes littering a chromosome of only 0.96 Mb (Darby et al. 2012). Ankyrin–repeat domain protein genes are reduced to just two, and the IS repertoire consists of six inactivated copies. When genomes from strains *w*Oo and *w*Di (a supergroup C symbiont from the dog heartworm, *D. immitis*) were compared with the supergroup D genomes (*w*Bm and *w*Ls), long–range conservation of gene order (synteny; Fig. 21.5) and pronounced GC skew were apparent only in the supergroup C genomes (Comandatore et al. 2015). Coupled with the almost complete loss of IS copies and heavy erosion of the replication, recombination and repair pathway in supergroup C, this clade exhibits signs of a longer co–evolutionary history with its hosts relative to supergroup D.

The presence of prophage genes in *Wolbachia* was known even before the first genomes were sequenced, and PCR surveys suggest that 89 % of *Wolbachia* strains in supergroups A and B harbour prophage WO (Kent and Bordenstein 2010). Furthermore, evidence of a lytic cycle producing phage virions has been obtained from several arthropod hosts, including *D. melanogaster* and *C. pipiens*. Phage WO has a genome composed of double–stranded, circular DNA and the icosahedral virions have been estimated at 20–40 nm in diameter. The association of phage WO with arthropod strains that can mediate reproductive manipulations, and the absence of prophages from the mutualistic genomes of supergroups C, D, and F, suggests that the phage may be important or even essential for the induction of CI and related phenotypes (Sinkins et al. 2005). This hypothesis has been bolstered by the recent discovery that the genome of *w*Rec, a CI–inducing strain from *Drosophila recens*, contains 33 prophage genes despite lacking the canonical minor capsid gene used to screen *Wolbachia* for the presence of phage WO (Metcalf et al. 2014). In conjunction with the finding that *w*Mel and *w*Au (the latter strain is incapable of mediating CI; see above) show key differences in prophage regions, including in candidate genes for CI, the potential role of phage WO in the most famous phenotypes of *Wolbachia* is generating renewed interest (Sutton et al. 2014). Even if genes encoded within prophages turn out not to have a direct role in CI, phage WO could still modulate reproductive phenotypes by lysing *Wolbachia* and thereby affecting

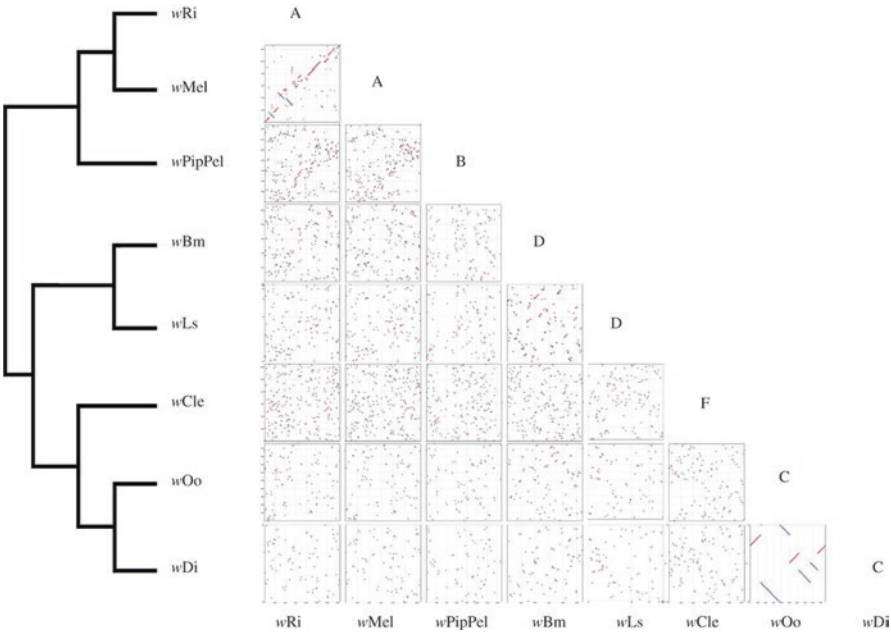

Fig. 21.5 Synteny conservation in supergroup C *Wolbachia*. A graphic representation of MUMmer v. 3.0 output is shown in the dot plots on the right. Red lines display collinear regions, whereas blue lines display inversions. Phylogenetic relationships among the *Wolbachia* strains are shown on the left. Reproduced from Comandatore *et al.* (2015) under a Creative Commons Attribution License http://creativecommons.org/licenses/by/4.0

symbiont densities, a scenario described as the "phage density model" (Bordenstein et al. 2006).

An elegant genetic selection study has recently provided important insights into the virulent phenotype of *w*MelPop in *D. melanogaster*, which had been one of the great unsolved mysteries in the *Wolbachia* field. A candidate region of eight genes, called Octomom, was identified as being subject to a series of tandem duplications in *w*MelPop (range, 2–10 copies of Octomom) (Chrostek and Teixeira 2015). By selecting *D. melanogaster* lines with different numbers of duplications, it was demonstrated that the density of *w*MelPop in fly tissues and the severity of the life–shortening phenotype were correlated with the degree of amplification of Octomom. Moreover, in one line, *w*MelPop reverted to a single copy of Octomom and was phenotypically indistinguishable from the closely related strain *w*MelCS_b, which also harbours one copy of Octomom and is non–pathogenic. These findings highlight the ability of *Wolbachia* to undergo rapid genome evolution.

Currently, our understanding of *Wolbachia* genomics is probably highly skewed due to the focus on comparisons between a limited diversity of arthropod strains (mainly from supergroups A and B) versus the C and D clades from filarial nematodes. However, many other *Wolbachia* supergroups in arthropods exist that lie outside the pandemic A and B clades, and these are often restricted to a single taxonomic

group. Partial genomes have recently been obtained for supergroup E and H strains, which are confined to springtails and termites, respectively. This enabled placement of supergroup E at the base of the current phylogenomic tree (Gerth et al. 2014), but further analyses of gene content in this and other host–specific clades might enable unique features of the pandemic strains to be identified. Other major gaps in our knowledge of *Wolbachia* genomes include the origin of the symbiosis in the plant–parasitic nematode, *R. similis* (Haegeman et al. 2009), and the relationship between supergroup F genomes from arthropods and filarial nematodes.

9 Lateral Gene Transfer

At one time the adzuki bean beetle, *Callosobruchus chinensis*, was believed to be co–infected with three distinct strains of *Wolbachia*. However, one of these strains could not induce CI and was not eliminated by antibiotic treatments, unlike the co–infecting symbionts. Intriguingly, genetic markers for this strain, designated *w*BruAus, were found at a twofold higher density in female beetles than their male counterparts (Kondo et al. 2002). Subsequent fluorescent in situ hybridisation studies demonstrated that "*w*BruAus" is actually a large (~380 kb) laterally transferred *Wolbachia* genome fragment that is integrated on the X chromosome of the host (Table 21.2) (Nikoh et al. 2008). The *Wolbachia* genes carried on this fragment were pseudogenised and transcriptionally silent. Discounting the numerous transfers of mitochondrial– and plastid–derived genes in eukaryotic genomes, this was the first report of lateral gene transfer (LGT) from an endosymbiont to its host.

It is now known that many, perhaps most, hosts of *Wolbachia* contain at least a small number of LGT fragments in the nuclear genome, and in some hosts, very substantial integrations of the *Wolbachia* chromosome have taken place (Table 21.2). The most extreme case reported to date is from *Drosophila ananassae*, in which multiple copies of the entire *w*Ana genome are present, probably ordered in a tandem array (Dunning Hotopp et al. 2007; Klasson et al. 2014; Choi et al. 2015). This "pseudogenome", which is largely transcriptionally inactive, constitutes ~2 % of the host nuclear genome and ~20 % of the heterochromatic chromosome 4. Analysis of various populations of *D. ananassae* worldwide has shown that this massive LGT is widely distributed, but appears to be unstable in some lines and can be lost (Klasson et al. 2014). *Wolbachia* LGT has also been identified in numerous filarial nematode genomes, including those of *B. malayi* (Ioannidis et al. 2013), *O. volvulus*, and *O. ochengi* (Fenn et al. 2006) (Table 21.2).

Clearly, the lifestyle of *Wolbachia* which involves an intimate association with the host germline is highly amenable to LGT. One key question raised by the high prevalence of *Wolbachia* LGT is whether functions encoded on the bacterial chromosome could become transferred to the host nuclear genome, perhaps even maintaining a key physiological role if the live symbiont infection was to be lost. Indeed, while non–functional nuclear mitochondrial DNA transfers are abundant in eukaryotes, it is also the case that ~1500 genes for mitochondrial proteins are

Table 21.2 Selected examples of *Wolbachia* lateral gene transfer into the host genome

Phylum	Species	Infection status[a]	Extent of lateral gene transfer	References
Nematoda	*Brugia malayi*	+	~115 kb derived from *w*Bm, including 32 intact ORFs	Ioannidis et al. (2013)
	Onchocerca flexuosa	–	178 *Wolbachia*–like fragments in genome; 97 *Wolbachia*–like genes in transcriptome	McNulty et al. (2010); McNulty et al. (2012)
	Onchocerca volvulus and Onchocerca ochengi	+	Two *Wolbachia* insertions of 100–200 bp each, containing indels	Fenn et al. (2006)
	Loa loa	–	15 degraded *Wolbachia*–like fragments of 33–188 bp	Desjardins et al. (2013)
	Acanthocheilonema viteae	–	49 *Wolbachia*–like fragments, including at least 28 intact ORFs	McNulty et al. (2010)
	Dictyocaulus viviparus	–	1,580 ORFs among ~1 Mb of *Wolbachia*–like sequence	Koutsovoulos et al. (2014)
Arthropoda	*Drosophila ananassae*	+	Up to 5 Mb of *Wolbachia*–like sequence, probably in a tandem array	Klasson et al. (2014); Choi et al. (2015)
	Callosobruchus chinensis	+	~380 kb of *Wolbachia* sequence transferred to the X chromosome	Kondo et al. (2002); Nikoh et al. (2008)
	Armadillidium vulgare	+	Three *Wolbachia* insertions spanning ~16 kb	Martin et al. (2010)
	Monochamus alternatus	–	At least ~30 genes transferred to an autosome	Aikawa et al. (2009)
	Nasonia spp.	+	Transfer of 13 *Wolbachia* ANK–PRANC domain genes	Werren et al. (2010)
	Glossina morsitans morsitans	+	Two *Wolbachia* insertions of 528 and 484 kb	Brelsfoard et al. (2014)
	Acyrthosiphon pisum	–	Expression of a LD–carboxypeptidase gene in the bacteriocyte derived from a *Wolbachia*–like ancestor	Nikoh and Nakabachi (2009)
	Chorthippus parallelus	+	Transfers of 448 and 144 kb of *Wolbachia* DNA from two different supergroups	Funkhouser–Jones et al. (2015)
	Aedes albopictus	+	Transfer and expression of *Wolbachia* transcriptional regulator WP0273	Hou et al. (2014)

[a]Presence or absence of live, cytoplasmic *Wolbachia* infection in extant populations

encoded by the nuclear genome of mammals. This question is pertinent, because several filarial nematode genomes from species that lack live, cytoplasmic infections harbour *Wolbachia* genes acquired by LGT. If these genes are functional, it might explain why the majority of filarial nematodes can thrive in a state of aposymbiosis. For instance, *Onchocerca flexuosa* is the only known species within the genus that is not infected with *Wolbachia*, but its genome is littered with an estimated 178 *Wolbachia*–like fragments (McNulty et al. 2010, 2012). Transcriptomic analysis of adult worms revealed 97 *Wolbachia*–like transcripts, and shotgun mass spectrometry detected peptides from two putative *Wolbachia* ABC transporters (McNulty et al. 2012). Follow–up studies showed tissue–specific expression patterns for some *Wolbachia*–like transcripts, including predicted metabolic enzymes, which were localised in similar regions to those occupied by *Wolbachia* endobacteria in other *Onchocerca* spp. (McNulty et al. 2013).

While these findings are certainly intriguing, and suggest that *O. flexuosa* might have been able to compensate for loss of *Wolbachia* by gene capture, this is probably not a model that applies to many other *Wolbachia*–negative filarial nematodes. Thus, the human parasite *Loa loa* lacks *Wolbachia* but contains just a few short, degraded LGT fragments in its genome (Desjardins et al. 2013). Furthermore, the *Loa loa* genome does not appear to have an expanded metabolic capacity or a larger repertoire of transporters for cofactors and vitamins that could explain its independence from *Wolbachia*. In conjunction with the discovery of apparent horizontal transmission of supergroup F symbionts in the evolutionary past of *Cercopithifilaria japonica* and *Mansonella* (*Cutifilaria*) *perforata* (Ferri et al. 2011), the hypothesis that *Wolbachia* are strictly obligate mutualists in filariae could be considered overly simplistic, and is certainly conditional on which particular *Wolbachia* clades are under consideration.

Are there clear examples of *Wolbachia* LGT leading to a newly encoded function in the host nuclear genome? There are perhaps only two cases with strong support. In the pea aphid, *Acyrthosiphon pisum*, an *ldcA* gene (encoding LD–carboxypeptidase) has been transferred into the aphid genome from a *Wolbachia*–like ancestor (although cytoplasmic *Wolbachia* is not currently present in *A. pisum*). The gene is highly expressed in the bacteriome and may be involved in recycling peptidoglycan on behalf of *Buchnera*, the primary endosymbiont of *A. pisum*, which contains peptidoglycan in its cell wall but lacks its own copy of *ldcA* (Nikoh and Nakabachi 2009). The second case involves a much more substantial LGT of three insertions spanning an estimated ~16 kb in *Armadillidium vulgare*. These insertions, referred to as the "F factor", contain numerous genes from prophage WO and are capable of mediating feminisation of *A. vulgare* in the absence of a live *Wolbachia* infection (Martin et al. 2010).

Wolbachia LGT can provide fascinating insights into the "ghosts of symbioses past", that is, the evolutionary history of extinct host–symbiont relationships. The suborder Rhabditina is a large taxon of nematodes that contains *Caenorhabditis* spp. alongside numerous animal parasites of medical and veterinary importance, but it is not known to contain any *Wolbachia*–infected species. Following genome sequencing of the rhabditine nematode *Dictyocaulus viviparus* (the cattle lungworm), ~1 Mb

of partially degraded *Wolbachia*–like sequences were discovered, and phylogenetic analysis of selected genes placed this "fossil" *Wolbachia* in supergroup F (Koutsovoulos et al. 2014). Several isolates of D. viviparus have been sequenced or subjected to targeted PCR and no live *Wolbachia* infections have been uncovered in this species, which lacks an arthropod vector. Interestingly, prophage WO sequences were well represented in the *Wolbachia* LGT of *D. viviparus*, indicating that clade F *Wolbachia* (including, perhaps, symbionts of filarial nematodes) may be infected with phage, despite the lack of prophages in the *w*Cle (bedbug symbiont) genome (Nikoh et al. 2014; Koutsovoulos et al. 2014).

10 Mammalian Immune Response

The discovery of *Wolbachia* in filarial nematodes raised an immediate and potentially clinically important question: are the endobacteria recognised by the host immune response, and if so, does this response contribute to the immunopathology observed in filarial diseases? The fact that *Wolbachia* are visible to the mammalian immune response was determined rapidly, although the first natural system examined was not humans harbouring filarial worms, but cats infected with *D. immitis*, which were shown to produce antibodies against *Wolbachia* surface protein (WSP) (Bazzocchi et al. 2000). Subsequently, antibodies to various *Wolbachia* proteins were detected in human filariasis patients, and the highest antibody levels to WSP and a heat–shock protein (GroEL) were observed in individuals infected with *Wuchereria bancrofti* and presenting with clinical signs of chronic lymphatic pathology (Punkosdy et al. 2003; Suba et al. 2007). In both lymphatic filariasis and onchocerciasis patients, chemotherapy is associated with release of *Wolbachia* (and symbiont DNA) into the blood (Keiser et al. 2002; Cross et al. 2001). Furthermore, in onchocerciasis patients treated with ivermectin, adverse reaction scores and serum concentrations of two neutrophil products (calprotectin and calgranulin B) were significantly correlated with levels of *Wolbachia* DNA (Keiser et al. 2002).

Wolbachia also has a key role to play in the ocular pathology of onchocerciasis. In a mouse model of keratitis, injection of untreated *O. volvulus* worm extracts induced more severe stromal thickening and stromal haze than did doxycycline–treated (*Wolbachia*–depleted) worm extracts (Saint André et al. 2002). Similarly, corneal pathology was increased when extracts of *B. malayi* were used rather than a preparation from *Acanthocheilonema viteae*, an aposymbiotic filaria. The severity of stromal haze was associated with the degree of neutrophil infiltration into the cornea. Importantly, this was consistent with earlier findings showing that *Wolbachia* were responsible for the neutrophilia within *O. volvulus* nodules, alongside a corresponding absence of neutrophils in nodules containing *O. flexuosa* (the aposymbiotic member of the genus, which parasitises red deer) (Brattig et al. 2001). Parallel observations regarding the dependence of neutrophil infiltration on *Wolbachia* were made following histological comparisons between *O. ochengi* nodules obtained from untreated and oxytetracycline–treated cattle (Nfon et al. 2006).

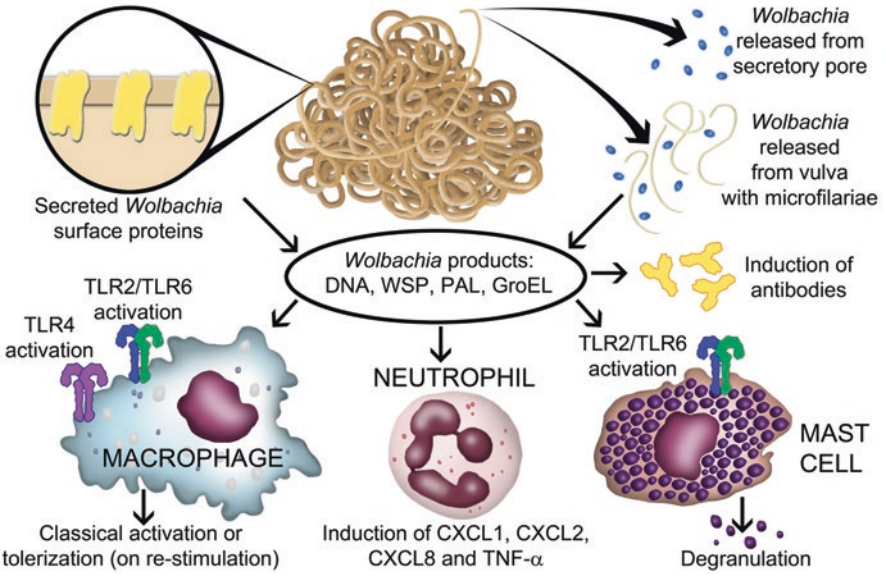

Fig. 21.6 Interaction of *Wolbachia* from filarial nematodes with the mammalian immune system. *Wolbachia* and *Wolbachia*–derived products [including *Wolbachia* surface protein (WSP), heat–shock protein GroEL, and peptidoglycan–associated lipoprotein (PAL)] are released from filarial nematodes via various routes. In addition, some *Wolbachia* proteins may be secreted into nematode tissues and become exposed on the cuticle. Through the activation of Toll–like receptor (TLR)–4 and/or TLR–2/TLR–6 heterodimers, macrophages, neutrophils, and mast cells respond to *Wolbachia* products and may release proinflammatory cytokines or degranulate. In the case of *Onchocerca ochengi*, neutrophils attracted by *Wolbachia* protect the adult worms from eosinophil degranulation, whereas in *Litomosoides sigmodontis* infection, *Wolbachia* facilitates migration of infective larvae by stimulating histamine release via mast cell degranulation

Wolbachia and *Wolbachia*–derived products can be released from filarial nematodes by a number of different routes, which are supported by varying levels of experimental evidence (Fig. 21.6). The most obvious process that could lead to release of endosymbionts is death of the nematodes, whether drug–induced or natural. Since parasite death caused by chemotherapy is much more acute than natural attrition, it is not surprising that drugs with a direct mode of action against filariae tend to be associated with potentially severe side effects, particularly in some individuals (who may have high worm burdens and/or a genetic predisposition to proinflammatory immune responses). However, numerous lines of evidence indicate that live adult worms also release *Wolbachia* and *Wolbachia*–derived molecules into surrounding tissues. First, *Wolbachia* has been observed in degenerating embryos and microfilariae released into nodule tissue by adult female *O. volvulus*, and the symbionts are likely to be released free in uterine fluid due to the relatively high rate of abnormal embryonic development in filariae, even in the absence of chemotherapy (Brattig et al. 2004; Kozek 2005). Second, free *Wolbachia* have been detected in the secretory–excretory canal of *B. malayi*, suggesting that small numbers of bacteria

could be liberated from the host via this route (Landmann et al. 2010). Finally, WSP family members have been immunolocalised in *B. malayi* tissues that lack symbionts, such as the cuticle (Melnikow et al. 2011), and have been detected on the adult female worm surface in *L. sigmodontis* (Armstrong et al. 2014). These findings show that filarial worms harbouring *Wolbachia* will partially resemble a bacterial infection to the immune system, although the lack of proliferation of *Wolbachia* in mammalian tissues prevents the more serious symptoms of bacterial infections, such as abscess formation and sepsis.

The identity of the key *Wolbachia* molecules that interact with the mammalian innate immune system, and the specific pathogen recognition receptors that bind them have been objects of some controversy. Shortly after *Wolbachia* were identified in filariae, it was claimed that lipopolysaccharide (LPS)–like molecules acting via CD14 and Toll–like receptor (TLR)–4 on monocytes and macrophages were responsible for proinflammatory immune reactions to *Wolbachia*–containing filarial extracts (Brattig et al. 2000; Taylor et al. 2000). However, *Wolbachia* genomes were subsequently shown to lack the biosynthetic capability to synthesise LPS (Wu et al. 2004), and the original data were revealed to be compromised by LPS contamination of parasite material (Turner et al. 2009). Nevertheless, recombinant WSP was reported to stimulate the production of proinflammatory cytokines [tumour necrosis factor–α, CXCL8, and interleukin (IL)–12] from blood cultures derived from *O. volvulus* patients, and responses to WSP were dependent on TLR–2 and TLR–4 (Brattig et al. 2004). Parallel studies have demonstrated a key role for WSP in the release of CXCL8 from neutrophils, as well as stimulation of chemokinesis and inhibition of apoptosis in these cells (Bazzocchi et al. 2003, 2007).

In contrast with these findings, a series of other studies have found no role for TLR–4 in the immune response to *Wolbachia*, but a dependency on TLR–2 (in complex with TLR–6) has been confirmed. For instance, in the mouse keratitis model, neutrophil infiltration, stromal haze, and release of the chemokines CXCL1 and CXCL2 require TLR–2 (Gillette–Ferguson et al. 2007). This pattern of TLR restriction suggests that diacylated lipoproteins are the main ligand present in *Wolbachia*–containing extracts, as these signal via TLR–2/6 heterodimers (Turner et al. 2009). Only a single lipoprotein with a high probability of surface expression, peptidoglycan–associated lipoprotein (PAL), was predicted from the *w*Bm genome. Strikingly, a synthetic diacylated N–terminal peptide from PAL could recapitulate all of the immunological effects of *Wolbachia*–containing filarial extracts, including dose–dependent induction of corneal haze and neutrophil infiltration in the mouse keratitis model. Furthermore, the synthetic peptide stimulated the production of proinflammatory mediators in monocyte cultures, and supernatants from these cells upregulated markers of inflammation on human lymphatic endothelial cells, suggesting a possible role for PAL in the pathology of lymphatic filariasis (Turner et al. 2009). Subsequent proteomic studies have shown that PAL is highly expressed in both *w*Bm and *w*Oo (Bennuru et al. 2011; Darby et al. 2012), although four and seven additional lipoproteins, respectively, are predicted from the genomes of these strains, which may also contribute to induction of the innate immune response to *Wolbachia* (Darby et al. 2012).

The most abundant protein in the *Wolbachia* proteome is not a lipoprotein, but heat–shock protein GroEL (Darby et al. 2012). Surprisingly, the role of this protein in the immune response to *Wolbachia* has been relatively neglected until recently. However, recombinant GroEL has been shown to induce apoptosis and senescence in monocytes from individuals exposed to *W. bancrofti* (Kamalakannan et al. 2015). In a further apparent contradiction to previous findings, GroEL was reported to interact with TLR–4, which is plausible since GroEL from other bacterial pathogens can signal via this receptor (Argueta et al. 2006). In summary, three of the most highly expressed proteins in *Wolbachia*—WSP, PAL, and GroEL—stimulate immune responses in filarial infections (Fig. 21.6), but the precise role of each in initial interactions with pathogen recognition receptors remains to be clarified.

A key evolutionary question relating to the filarial–*Wolbachia* symbiosis is whether the symbiont–induced immune responses are beneficial, detrimental, or effectively neutral to the nematode host. Of course, the answer to this question is likely to differ depending on the filarial species involved, which display significant life cycle differences, such as the preferred anatomical location of the adult worms and microfilariae in the host. In the case of the nodule–forming *Onchocerca* spp., such as *O. volvulus* and *O. ochengi*, the sustained infiltration of neutrophils into the nodule might be considered likely to damage the worms. However, these species can live for about a decade without suffering any obvious damage from this persistent neutrophilia. In *O. ochengi* infection in cattle, prolonged oxytetracycline treatment leads to abatement of the neutrophil influx and a gradual replacement of these cells with eosinophils, which are well equipped to target helminth infections (Nfon et al. 2006). In the absence of neutrophils, the eosinophils degranulate on the adult worm cuticle, causing structural damage, and several months later the worms eventually die. This specific pattern of eosinophil degranulation preceding worm death is not observed when cattle are treated with an anthelminthic drug that directly targets the worms without affecting *Wolbachia* (Hansen et al. 2011). Thus, *w*Oo has apparently become a defensive mutualist that misdirects the mammalian immune response, presenting a bacterial "signature" that recruits an inappropriate effector cell, the neutrophil. Through a currently unknown mechanism these cells, which are incapable of phagocytosing *Wolbachia* beneath the nematode cuticle, form a barrier and prevent an eosinophil response from eliminating the worms. Interestingly, a second case of *Wolbachia* facilitating filarial infection has been reported from *L. sigmodontis*, in which TLR–2–dependent stimulation of mast cells leads to degranulation, an increase in vascular permeability, and improved migration of infective larvae through host tissues (Fig. 21.6) (Specht et al. 2011).

11 Use in Vector Control

Attempts to control disease vectors using *Wolbachia* have a long history. In 1967, the World Health Organisation sponsored a project to control lymphatic filariasis in which several waves of male *C. quinquefasciatus* carrying a *Wolbachia* strain which was incompatible with that in the local population were released in a village near

Rangoon, Burma. The mosquito population was successfully suppressed within 12 weeks (Laven 1967). Despite this early positive experience, it is only relatively recently that attempts to control vector–borne diseases using this "Incompatible Insect Technique" (Fig. 21.7) have been resurrected. For instance, Aedes polynesiensis is a highly efficient vector of lymphatic filariasis in the South Pacific and is extremely difficult to control using insecticides due to its ability to exploit cryptic and inaccessible breeding sites. This species is naturally infected with a supergroup A *Wolbachia* strain, but replacement of this by introgression with *Aedes riversi* carrying a supergroup B strain leads to production of *Ae. polynesiensis* males that are incompatible with wild *Ae. polynesiensis* females (Brelsfoard et al. 2008). Trials in which *Ae. polynesiensis* males carrying the *Ae. riversi Wolbachia* strain were released on a small scale on uninhabited islands of French Polynesia showed suppressive effects on the wild mosquito population, and no accidental release of infected females (which could lead to unintended population replacement) occurred (O'Connor et al. 2012). A similar approach has been proposed for control of *C. quinquefasciatus* across islands of the southwest Indian Ocean, where this species is a major vector of Rift Valley Fever virus and lymphatic filariasis. Here, the wPip(Is) strain from *C. pipiens* f. *pipiens* obtained from Turkey was introgressed into the nuclear background of *C. quinquefasciatus* from La Réunion (Atyame et al. 2011). Males carrying the *w*Pip(Is) strain were found to be incompatible with females naturally infected with *w*Pip(LR), and these wPip(Is) males were equally competitive with *w*Pip(LR) males in cage experiments, leading to a population crash. These promising trials for control of *Ae. polynesiensis* and *C. quinquefasciatus* provide grounds for hope that the incompatible insect technique will be used ultimately on a large scale as an environmentally friendly method to suppress natural mosquito populations across island habitats.

The discovery that *Wolbachia* prevent the transmission of pathogens by vectors has led to a revolutionary alternative approach to vector control, the Population Replacement Technique (Fig. 21.7). The main focus of this approach to date has been on controlling the spread of dengue by releasing *Wolbachia*–infected *Ae. aegypti*, which are refractory for dengue virus and spread through natural populations (which lack *Wolbachia*) via strong CI. Initially, *Ae. aegypti* was transfected with *w*MelPop, which has a threefold effect on this species: blockage of dengue transmission, induction of CI, and reduction of adult lifespan by 50 % (which also reduces vector competence due to the long extrinsic incubation period of dengue virus) (McMeniman et al. 2009; Moreira et al. 2009). While this combination of deleterious effects would seem to render *w*MelPop the strain of choice for release into the wild, it also has other significant fitness effects on *Ae. aegypti*, including a reduction in the viability of diapausing and non–diapausing eggs (Yeap et al. 2011). The impact on the viability of diapausing eggs was particularly strong when the eggs were stored for a few weeks, suggesting that *w*MelPop might fail to spread through wild populations of *Ae. aegypti* when conditions were dry.

These considerations led to concerted efforts to transfect *Ae. aegypti* with the non–pathogenic *Wolbachia* strain from *D. melanogaster*, *w*Mel, which was achieved following passage of *w*Mel in a mosquito cell line for 2 years prior to microinjection into *Ae. aegypti* embryos (Walker et al. 2011). Although *w*Mel

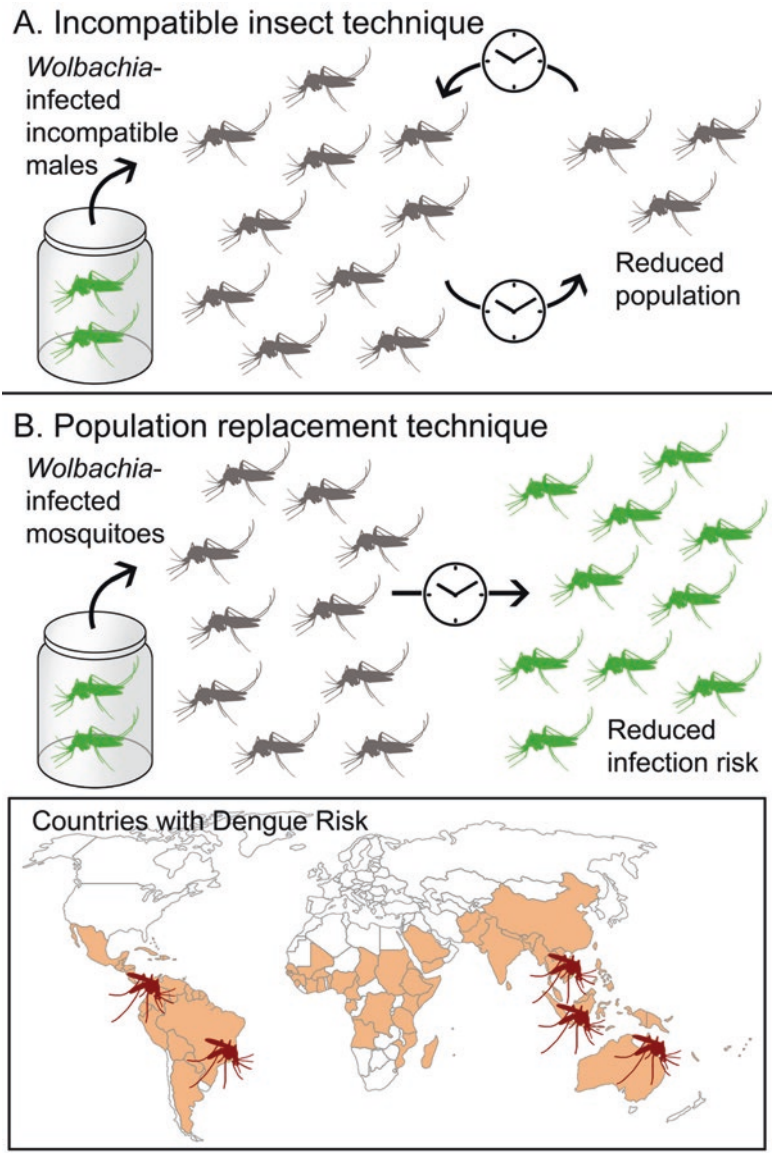

Fig. 21.7 Techniques for the exploitation of *Wolbachia*–induced cytoplasmic incompatibility (CI) for the control of mosquito–borne diseases. (**a**) In the incompatible insect technique, males infected with non–native *Wolbachia* are released into the wild population. Released males that mate with wild females fail to produce offspring, reducing the population's reproductive success and thereby reducing mosquito numbers; (**b**) Population replacement relies on the ability of *Wolbachia* to spread through populations through CI. Mosquitoes containing a *Wolbachia* strain that suppresses disease transmission are introduced into the wild population. Population replacement is being trialled at various sites by the International Eliminate Dengue Project with the aim of reducing dengue fever transmission by targeting the mosquito *Aedes aegypti*, the main vector. The *inset* shows countries that were endemic or probably endemic for dengue fever in 2014 shaded in *orange* (source: CDC), with trial release sites indicated by *mosquito symbols* (source: www.eliminatedengue.com)

attained much lower densities in mosquito tissues compared with *w*MelPop, the former still induced strong CI and completely blocked the secretion of dengue virus into saliva. Moreover, *w*Mel had no discernible fitness effects on *Ae. aegypti*, since both adult longevity and egg viability remained normal. In cage experiments under semi–field conditions, *w*Mel outperformed *w*MelPop by reaching fixation in the mosquito population more rapidly (Walker et al. 2011). Subsequently, field releases of *Ae. aegypti* infected with either strain were undertaken in different isolated sites near Cairns, Queensland, Australia. In accordance with the cage experiments, the spread of *w*Mel through natural populations of *Ae. aegypti* was more successful than for *w*MelPop, with near–fixation reached in two suburbs approximately 5 weeks after 10 releases of ~15,000 *w*Mel–infected mosquitoes had been completed (Hoffmann et al. 2011). Relatively small–scale releases of *Wolbachia*–infected *Ae. aegypti* have also taken place in Tri Nguyen Island, Vietnam; Yogyakarta, Indonesia; Bello, Colombia; and Rio de Janeiro, Brazil (Fig. 21.7). The first trial involving releases on a citywide scale began in October 2014 in Townsville, Queensland, and is ongoing. Recently, dissemination of *Wolbachia*–infected *Ae. aegypti* has also been proposed as a key strategy to control the spread of the Zika fever epidemic in South America, since strain wMel can drastically reduce Zika virus dissemination in *Ae. aegypti* and blocks appearance of infectious viral particles in the saliva (Dutra et al. 2016).

While this approach shows great promise for the control of dengue and other arboviruses, it is too early to judge whether population replacement with *Wolbachia*–infected *Ae. aegypti* will ultimately reverse the dramatic spread of dengue observed over the past 50 years. However, *w*Mel–infected *Ae. aegypti* captured near Cairns 1 year after the initial releases remained refractory to dengue virus when challenged in the laboratory, indicating that the pathogen–blocking phenotype is stable, at least in the short term (Frentiu et al. 2014). A counterbalancing argument is the enhanced vector competence conferred by wPip in the natural *C. quinquefasciatus–P. relictum* system (Zele et al. 2014), and the facilitation of West Nile virus infection in Culex tarsalis (a naturally *Wolbachia*–free species) transiently transfected with strain *w*AlbB (Dodson et al. 2014). These examples of pathogen enhancement suggest that all major pathogen–vector pairs should be tested for the impact of *Wolbachia* infection in the laboratory before *Wolbachia*–infected vectors are released on a large scale into communities.

12 Susceptibility to Antibiotics

The susceptibility of *Wolbachia* to antibiotic treatment was assessed rapidly after its definitive identification in filarial nematodes, using a variety of in vitro (mosquito or *Drosophila* cell lines naturally or artificially infected with *Wolbachia*, and adult filarial nematode cultures) and in vivo (*B. malayi* in jirds, *L. sigmodontis* in mice, and *O. ochengi* in cattle) screens. Prophylactic effects of tetracyclines against filarial nematodes were recognised even before the discovery of *Wolbachia* (Bosshardt

et al. 1993), and the efficacy of several members of the tetracycline class (tetracycline, oxytetracycline, and doxycycline) against *Wolbachia* was appreciated at an early stage. In small animal models, several weeks of tetracycline treatment resulted in growth retardation of developing worms or sterilisation of adult females if therapy commenced after establishment of patent infection (Bandi et al. 1999; Hoerauf et al. 1999). However, in the *O. ochengi*–cattle system, intermittent oxytetracycline treatment over a period of 6 months led to killing of all adult worms (Langworthy et al. 2000), whereas continuous chemotherapy over 2 weeks was neither adulticidal nor embryostatic and permitted recrudescence of *Wolbachia* in worm tissues (Gilbert et al. 2005).

The first human trials of antibiotics against onchocerciasis used 100 mg doxycycline daily for 6 weeks, and showed apparently irreversible sterilisation of adult female worms leading to suppression of microfilariae in the skin (Hoerauf et al. 2001; Hoerauf et al. 2000a). Follow–up studies demonstrated that an increase of the dose to 200 mg per day killed >60 % of adult female *O. volvulus* (Hoerauf et al. 2008a). However, a recent meta–analysis indicated that all clinical trials of doxycycline performed to date, including one in which 200 mg per day was administered for just 4 weeks, were statistically indistinguishable in terms of efficacy and reduced adult female worm lifespan by 70–80 % (i.e. from ~10 years to 2–3 years) (Walker et al. 2015). These results are remarkable considering that no other safe, adulticidal drug for onchocerciasis currently exists. Notwithstanding the clear efficacy of doxycycline and its successful use in a large, community–level trial in which very high rates of compliance and therapeutic coverage were achieved (Tamarozzi et al. 2012; Wanji et al. 2009), it is not approved for mass drug administration because of (a) the long treatment regimen and (b) contraindications in pregnant or breastfeeding women and children aged less than 8 years. Doxycycline has shown equally impressive efficacy in lymphatic filariasis, killing 80 % of adult worms following a regimen of 200 mg per day for 4 weeks (Debrah et al. 2007); moreover, a 6–week course has the additional major benefit of ameliorating the pathology of the disease (both lymphedema and hydrocele) (Debrah et al. 2006). Whether this latter effect is due to the antibacterial action of doxycycline or its intrinsic anti–inflammatory activity has not yet been fully resolved. As for onchocerciasis, doxycycline can be used for the treatment of individual lymphatic filariasis patients (Table 21.3), but has not been incorporated into mass preventive chemotherapy programmes (Taylor et al. 2010).

The “Holy Grail” of adulticidal drug development for filarial diseases is the identification of a chemotherapeutic regimen that is safe and effective following a course of 7 days or less (Taylor et al. 2014). An antibiotic with fewer contraindications than doxycycline and equivalent or superior efficacy in vitro and in small animal models is rifampicin (Table 21.3) (Hermans et al. 2001; Fenollar et al. 2003; Volkmann et al. 2003; Townson et al. 2006). However, in human trials, rifampicin alone has not given grounds for radically shortening antibiotic regimens for onchocerciasis, as 5–day and 2–week continuous regimens are completely ineffective (Richards et al. 2007; Specht et al. 2008), whereas a 4–week regimen only achieved a borderline effect on embryogenesis (Specht et al. 2008). In lymphatic filariasis, a 2–week combination regimen of doxycycline and rifampicin induced moderate adulticidal activity, but was apparently less efficacious than a 4–week course of doxycycline (Debrah et al. 2011).

Table 21.3 Compounds with activity against *Wolbachia* and current or potential use in the treatment of filarial infections

Drug	Class	Status	Notes[a]	Key references or trial ID[b]
Doxycycline	Tetracycline	Recommended for individual treatment of onchocerciasis or lymphatic filariasis	Not approved for mass drug administration due to long regimen and contraindications in children under 8 and pregnant/breastfeeding women	Hoerauf et al. (2000a, 2008a); Taylor et al. (2005); Debrah et al. (2007)
Rifampicin	Rifamycin	Has been trialled for lymphatic filariasis and onchocerciasis	Some evidence for adulticidal activity in combination with doxycycline for lymphatic filariasis; latest trials evaluate efficacy in combination with doxycycline for onchocerciasis	Specht et al. (2008); Richards et al. (2007); Bah et al. (2014); Debrah et al. (2011); ISRCTN68861628
Azithromycin	Macrolide	Has been trialled alone and in combination with rifampicin for onchocerciasis	Very low efficacy; unlikely to be evaluated further	Richards et al. (2007); Hoerauf et al. (2008b)
Moxifloxacin	Fluoroquinolone	Currently in clinical trials in combination with rifapentine for onchocerciasis	Shows good activity against adult *Onchocerca gutturosa* in vitro and *Litomosoides sigmodontis* in vivo	Johnston et al. (2014); ISRCTN43697583
Rifapentine	Rifamycin	Currently in clinical trials in combination with moxifloxacin for onchocerciasis	Shows good activity against adult *Onchocerca gutturosa* in vitro and *L. sigmodontis* in vivo	Johnston et al. (2014)
Minocycline	Tetracycline	Currently in clinical trials for onchocerciasis (compared with doxycycline or albendazole)	Consistently displays superior activity to doxycycline in both in vitro and in vivo screens; reported higher incidence of adverse reactions compared to doxycycline	Townson et al. (2006); Johnston et al. (2014); ISRCTN06010453
Globomycin	LspA inhibitor	Investigational drug only (not commercially available)	Shows some activity against adult *B. malayi* in vitro, leading to reduced motility and viability	Johnston et al. (2010)

(continued)

Table 21.3 (continued)

Drug	Class	Status	Notes[a]	Key references or trial ID[b]
wALADin1	Benzimidazole	Investigational drug only (current compounds insufficiently potent for in vivo use)	A selective inhibitor of *Wolbachia* δ–aminolevulinic acid dehydratase that can kill adult *B. malayi* and *L. sigmodontis* in vitro	Lentz et al. (2013)
Berberine	Isoquinoline alkaloid	Investigational drug only (insufficiently potent for in vivo use)	An inhibitor of the GTPase activity of the *Wolbachia* cell division protein FtsZ; reduces motility and microfilarial production in adult *B. malayi*	Li et al. (2011)
Corallopyronin A	Corallopyronin	Investigational drug only (natural product; currently biosynthesis is too costly for preclinical trials)	A non–competitive inhibitor of bacterial DNA–dependent RNA polymerase that depletes *Wolbachia* and impedes development of *L. sigmodontis* in vivo	Schiefer et al. (2012)
Albendazole sulphone	Benzimidazole metabolite	Approved for mass drug administration of lymphatic filariasis; currently in clinical trials (in combination with doxycycline) for onchocerciasis	Interferes with binary fission of *Wolbachia* in *B. malayi* ovaries independently of activity against nematode microtubules (unclear if anti–*Wolbachia* activity is achievable at physiologically relevant concentrations)	Serbus et al. (2012); ISRCTN06010453
Acyldepsipeptides	ClpP dysregulator	Investigational drug only (further optimisation of stability and activity required)	Dysregulates ClpP peptidase activity, leading to promiscuous degradation of *Wolbachia* FtsZ and other proteins, and killing of adult *L. sigmodontis* in vitro	Schiefer et al. (2013)
Fosfomycin	Phosphonic acid derivative	Licensed for other indications, but unclear if anti–*Wolbachia* activity can be achieved safely in vivo	Blocks lipid II synthesis needed for cell division (enzymes essential for making peptidoglycan are not present in the *Wolbachia* genome)	Vollmer et al. (2013)

[a]We are grateful to Dr Kenneth Pfarr (University of Bonn) for providing additional information for this table

[b]Obtained from the ISRCTN trial registry at http://www.isrctn.com

This outcome led to a new clinical trial of a 3–week combination of doxycycline and rifampicin for the treatment of onchocerciasis, the results of which are pending (Table 21.3). In the interim, a 3–week combination regimen of oxytetracycline and rifampicin was evaluated alongside 3–week courses of either drug alone against *O. ochengi* in cattle. Surprisingly, the combination regimen exhibited lower efficacy in terms of both adult worm burden and microfilarial load compared with 3 weeks of oxytetracycline only, while 3 weeks of rifampicin alone was completely ineffective (Bah et al. 2014). The poor performance of the combination treatment was hypothesised to be caused by pharmacokinetic interference between the two drugs, which has been reported for other tetracycline–rifampicin combinations used for chemotherapy of bacterial infections in humans.

Outside the tetracycline and rifamycin classes of antibiotics, success in identifying other licensed drugs with activity against *Wolbachia* has been limited. In early screens in vitro and in small animal models, antibiotic classes with efficacy against other *Rickettsiales* such as macrolides (e.g. erythromycin, azithromycin), fluoroquinolones (e.g. ciprofloxacin, ofloxacin, and levofloxacin), and chloramphenicol were shown to be ineffective (or weakly effective) at depleting *Wolbachia* (Fenollar et al. 2003; Hoerauf et al. 2000b). In the case of azithromycin, this has been confirmed further in two human trials (Table 21.3) (Richards et al. 2007; Hoerauf et al. 2008b). A benzimidazole metabolite, albendazole sulphone (Serbus et al. 2012), and a licensed phosphonic acid derivative, fosfomycin (Vollmer et al. 2013), have recently been shown to target *Wolbachia* effectively in vitro, but it remains unclear if sufficiently high concentrations of these drugs could be safely attained in vivo for significant anti–*Wolbachia* activity (Table 21.3). Subsequently, the Anti–*Wolbachia* Consortium (A·WOL) was established to screen and evaluate licensed and novel compounds, including drugs from the approved human drug pharmacopoeia with a potential to be repurposed (Johnston et al. 2014; Taylor et al. 2014). The consortium relied on a screening pipeline that progressed from a high–throughput mosquito cell line assay (strain *w*AlbB in C6/36 *Ae. albopictus* cells) through an in vitro adult *Onchocerca gutturosa* adult worm screen and finally to in vivo testing in the *L. sigmodontis* mouse model (Clare et al. 2015; Johnston et al. 2014). Unexpectedly, while additional licensed tetracyclines (e.g. minocycline) and rifamycins (e.g. rifapentine) were shown to have good activity in the A·WOL pipeline, the fluoroquinolone moxifloxacin was also prioritised due to consistently high efficacy (Johnston et al. 2014). Clinical trials are currently underway to evaluate a combination of rifapentine and moxifloxacin against onchocerciasis (Table 21.3).

The remarkable persistence of *Wolbachia* during antibiotic treatment in filarial hosts is perhaps surprising considering that significant barriers to drug penetration in these nematodes have not been identified. In contrast to the several weeks of antibiotic chemotherapy required for filarial infections, a 2–to–5 day course of doxycycline is highly effective for most rickettsial infections (Botelho–Nevers et al. 2012). However, in common with most obligate intracellular bacteria, *Wolbachia* lacks inheritable resistance mechanisms (McOrist 2000). During doxycycline treatment of a mosquito cell line in vitro, strain *w*MelPop–CLA (a cell line–adapted variant of *w*MelPop, which does not over–replicate) exhibited a suite of changes in mRNA and protein expression that were associated with antibiotic tolerance or

"metabolic resistance" (Darby et al. 2014). These included upregulation of core metabolic pathways for energy generation and nucleotide synthesis, and reduced expression of several outer membrane proteins, which is consistent with remodelling of the bacterial surface. Furthermore, levels of a phosphate ABC transporter ATPase (PstB) increased significantly, and this protein is known to be involved in drug efflux in free–living bacteria. These phenotypic adaptations, coupled with the location of *Wolbachia* in filarial worms where it is protected from clearance by the immune system, enable the symbionts to survive short–term antibiotic therapy and to recrudesce once drug pressure has been lifted (Gilbert et al. 2005). Nevertheless, *Wolbachia*'s mutualistic relationship with its filarial hosts may turn out to be its Achilles' heel, since it is not necessary to identify drug combinations that achieve complete eradication of *Wolbachia*, but only one that disrupts the symbiosis for a sufficient period of time such that the adult worms are irreversibly compromised.

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Index

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