REVIEW

Ehrlichioses and anaplasmoses: (re)emerging tickborne zoonoses in humans and in animals

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Introduction

One cannot but point out that the variety of topics related to zoonoses, and in particular zoonoses transmitted by ticks, is fascinating and ranges from environmental to clinical sciences, also in emergency situations, from veterinary sciences to human epidemiology, in a finely woven network of interactive ecological, social and health factors. Anthropic and cultural factors are correlated to the presence and distribution of zoonoses and, in most cases, become risk factors themselves. The epidemiology of zoonoses is, in fact, closely tied to the socio-economic conditions and the characteristics of the different rural areas, where complex interrelations and close living conditions exist between men and animals and are often unavoidable.
In recent years the situation has become even worse, making zoonoses one of the most serious problems of Public Health. The list of zoonoses is extensive and the agents responsible for infection include bacteria, fungi, chlamydiae, rickettsiae, viruses and protozoa, transmitted by faeces, food, animal bites or inoculations by arthropods. The role of arthropods is significant and is in constant expansion. Human activities modify the ecological balance and facilitate an increase in arthropods and, consequently, the spread of pathogens they transmit with their relative pathologies [1-3]. Because of the vast range of hosts among pets and wild animals, it is very difficult to control arthropods and their respective pathologies. The same tick may have different specificities of hosts, the same host may be parasitized by various species of ticks. Mammals, birds, and reptiles can be hosts for ticks, which transmit more pathogens than any other vector and can guarantee the transmission and continuance in nature even by transtadial or transovarial means. In recent years, moreover, the epidemiology of arthropods has changed: new forms of transmission have been described and new pathogens have been isolated [4-8].

Bioecology of ehrlichiae

Ehrlichiae are obligate, gram-negative, intracellular bacteria. Inside the cells the initial bodies and the morulae can be identified and can be stained with Romanowsky method (acidophilic staining), Gimenez method (azurophilic granules) or with acridine orange [9]. They are present in the blood in the acute phase and can be isolated from it. The developmental cycle takes place between mammals and arthropod vectors, ticks, which have also demonstrated transtadial transmission. Ecological and anthropic factors determine the distribution of arthropod vectors and their consequent, correlated pathologies. Geographic factors, the physical environment, the composition of the ground cover, the biodiversity of the flora, the hydrology and humidity of the ground, the distribution and density of the fauna that acts as a reservoir and other ecological variables can act as predictors to build probability models of the distribution of vectors and their pathogen hosts [10]. The role of wildlife in supporting the diffusion of zoonoses was recently described [11, 12]. The detection of the presence of antibody reactivity in human and animal serums is used to monitor the circulation of the pathogens and their vectors. In the USA the growth of the white-tailed deer, *Odocoileus virginianus* during the last century has been connected to the simultaneous increase of the population of *Amblyomma* and its pathogen hosts. The white-tailed deer is an essential host in the evolutive stages of *Amblyomma*, and acts as a reservoir for *Ehrlichia chaffeensis* (*E. chaffeensis*), *Ehrlichia ewingii* (*E. ewingii*), *Borrelia lonestari* and others [13-15]. The racoon (*Procyonis lotor*) is also present in the epidemiology of *Ehrlichia* [16]. *Rhipicephalus sanguineus* (*R. sanguineus*) is mainly responsible for the transmission of the agent *Ehrlichia canis* (*E. canis*), while *Amblyomma americanum* (*A. americanum* – lonestar tick) is responsible for the transmission of *Human Monocytic Ehrlichiosis* (HME) and of *Human Ehrlichiosis Ewingii* (HEE) agents. The transmission of *Human Granulocytotropic Anaplasmosis* (HGA) has as a vector the tick *Ixodes scapularis* (*I. scapularis* – black-legged tick) in the eastern USA, *Ixodes pacificus* (*I. pacificus*) along the US west coast and, prevalently, *Ixodes ricinus* (*I. ricinus*) in Europe. Other *Ixodidae* have also been included in the list of possible or suspected vectors of *Ehrlichia* and *Anaplasma*. *Anaplasma phagocytophilum* (*A. phagocytophilum*) was identified in *Ixodes exagonus* (5,9%) in the Netherlands (17), in *Ixodes persulcatus* (4%) in northeastern China [18], in Inner Mongolia and in the Heilongjiang Province [19], in Korea (1%)

with *E. chaffeensis* as well [20], in Japan (1%) [21], in eastern Europe (2,4%) [22]. This agent was also associated with *Ixodes spinipalpis* in the USA [23,24], *Ixodes trianguliceps* in the United Kingdom [25] and reported in *Ixodes ventalloi* in Portugal [26] and in *Ixodes dammini* removed from a patient with HGA [27]. *A. phagocytophilum* was detected in *Dermacentor silvarum* [18], in *Dermacentor reticulatus* (*D. reticulatus*) in Austria [28], in *Dermacentor variabilis* (*D. variabilis*) in California [29]. *D. reticulatus* was found to occur at far more sites than previously known in Germany [30] and was reported to be the major tick species attacking dogs in the Warsaw area [31]. The competency of *D. reticulatus* as a vector of *Anaplasma*, however, has yet to be demonstrated. Wild rodents were thought to be implicated in tick-rodent cycles and the prevalence of *A. phagocytophilum* in alternate ticks beside the known vector species was supposed to be attributable to a secondary maintenance cycle in relatively host specific, usually nonhuman-biting ticks and their hosts [32].

Canine ehrlichiosis *(E. canis, E. ewingii)*

E. canis and *E. ewingii*, belonging to the family *Anaplasmataceae*, genus *Ehrlichia*, are the agents respectively of *Canine Ehrlichiosis* (CE) and *Canine Granulocytic Ehrlichiosis* (CGE). *E. canis* circulates widely in dogs. Its vector is *R. sanguineus*. Symptoms of the illness observed clinically in dogs include fever, asthenia and anorexia, nasal discharge, red rheumy eyes, splenomegaly, lymphadenopathy, dyspnea. There is also damage to the liver, signs of haemorrhage, epistaxis, haematuria and melena, thrombocytopenia with hypogranulocytosis [33]. The infection was documented in the Mediterranean area, Israel, Egypt, Tunisia, Portugal, Spain, Greece [34-39], but also in Germany, Switzerland, UK, Sweden, Poland, The Netherlands [40-45] and in different parts of Africa, India, South-East Asia [46, 47]. In Italy, initially in Sicily and later in Sardinia and then in all of Italy: from 50 to 72% of the dogs examined tested positive; the mortality oscillated between 6 and 12% [48-50]. Army dogs in Senegal had a 49 to 85% positivity, 50% in Chad and 46% in Zimbabwe. Clinical and serological data from Israel and Morocco showed an elevated prevalence and an elevated mortality [51-53]. In Oklahoma from 3 to 6% of the dogs examined tested positive to PCR for *E. canis*, *E. ewingii*, *E. chaffeensis*. PCR positivity was also observed in various species of ticks (*R. sanguineus*, *D. variabilis*, *Anaplasma americanum)* [54]. In Sicily, antibody reactivity to *E. canis* was found in 55% of stray dogs and in 11% of pet dogs: these data evidenced an intense circulation of the agent in unprotected environments. On the contrary, human serums, taken from feverish patients and healthy blood donors, tested negative for *E. canis* and *E. chaffeensis*, despite the proved presence and the high density of the arthropod vector, *Rhipicephalus* [55]. Recent studies carried out in Brazil on haemorrhagic and thrombocytopenic dogs revealed an elevated incidence of *E. canis* infections, but also the presence of *Babesia* and *Anaplasma platys* [56], and in the south-east (Saõ Paolo) a study on a group of dogs showed an 80% positivity to PCR for *E. canis* [57]. An intense circulation of *E. canis* was found in Thailand and in Cameroon, revealing a considerable circulation of strains of *E. canis* but also of *E. ewingii*, identical in the genetic sequence to North American strains [58-60]. Studies conducted in Israel suggested that some symptoms observed in *E. canis* infections can be attributed to immune complexes and to the presence of antiplatelet antibodies [61]. A form of recurrent pyodermitis from positive coagulase *Staphylococcus intermedius*, linked to chronic Ehrlichiosis, has been described, especially in German shepherd [62].

Human ehrlichioses

Between the 1980s and early 2000s, new agents of the *Order Rickettsiales* were identified in the world. Some tick-borne species were isolated before their pathogenic role in men was recognized. *Rickettsia parkeri*, among others, was classified as a pathogen for men sixty years after it had been isolated in ticks. *Rickettsia felis*, a new *Rickettsia* in the Spotted Fever group, was associated with fleas as vectors and only recently was its pathogenicity for men identified [7, 8, 63]. *Rickettsia ambliommi* was identified as a possible cause of a rickettsiosis previously reported as Rocky Mountain Spotted Fever [64].
Ehrlichia and *Anaplasma*, gram-negative, intracellular, wide-spread micro-organisms, already classified in the *Rickettsiaceae* and considered to be of prevalent veterinarian interest were recently associated with numerous clinical evidences in men, revealing a diffusion in the human sphere which is much greater than previously believed [65].
Human *ehrlichiosis* and *anaplasmosis*, refer to infections with at least three different bacteria, obligate intracellular parasites, belonging to 2 genera of the family *Anaplasmataceae*: *E. chaffeensis*, *E. Ewingii*, two species of the genus *Ehrlichia*, *and A. phagocytophilum*, of the genus *Anaplasma*. A new species with strong tropism for mononuclear WBC was isolated in 1991 by a recruit with febrile syndrome at Fort Chaffee in Arkansas. The illness, called HME, was attributed to a new *Ehrlichia*: *E. chaffeensis* [66]. In 1994 another species identified in the morulae of granulocytes was isolated from patients in Minnesota and Wisconsin (in some cases resulting in death), believed to be similar to *Ehrlichia equi* (*E. equi*) or *Ehrlichia phagocytophila* (*E. phagocytophila*) and called *Human Granulocytotropic Ehrlichiosis* agent, today an *Anaplasmosis* (HGA), caused *by A. phagocytophilum* [67]. Since then nearly 4500 cases of HME have been reported in the southeastern United States and nearly 3000 cases of HGA in the western and northeastern USA. *E. ewingii* was, in 1999, recognized as agent of another human *Ehrlichiosis* (HEE) [68].The incidence of these infections is increasing constantly where the appropriate tick vectors

are found and is connected to the increase in arthropod populations. The diseases have non-specific clinical signs and important laboratory data (thrombocytopenia, leukopenia, hypertransaminasemia), common to the three infections. Dermatologic manifestations may help in sustaining a suspected diagnosis [69]. Infections are more prevalent and may have severe clinical picture and lethal outcome in immuno-compromised hosts, in particular in HIV infections [70]. The practical challenge is posed by the difficult diagnostic dilemma with the insurgence of clinical signs. The clinical suspicion of a tick bite and the orientation towards the possible infective agents becomes extremely important. Beginning antibiotic treatment promptly, when it is more efficacious and can resolve the situation, can help prevent undesirable consequences [71]. Human cases require for confirmation to be defined by some essential characteristic: fever with history of exposure to tick or tick bite; at least a ≥ 4 fold increase of antibody titre in acute and convalescent sera; positive PCR and sequencing of the amplicons demonstrating specific DNA or isolation of the agent from blood. A group of specialists and experts of the CDC in Atlanta, GA, have developed guidelines for health workers which focus on the practical aspects related to the diagnosis of tick-borne rickettsial diseases (*Rickettsiales Order*) and concerning the epidemiology, clinical approach, treatment and laboratory diagnosis [72]. In Europe, the ESCMID Study Group on *Coxiella*, *Anaplasma*, *Rickettsia* and *Bartonella*, has also published comprehensive guidelines for the diagnosis of tick-borne bacterial diseases, to help clinicians and microbiologists in diagnosing and have a better understanding of these infections [73].

HME, *E. chaffeensis*

After the initial findings at the beginning of the 1990s, as reported above, the number cases of HME reported to the CDC amounted to several thousand. In Georgia serological, cell culture and molecular identification tests demonstrated a prevalence of as high as 92% of infection in white-tailed deer (*Odocoileus virginianus*), but an elevated prevalence was also shown in racoons (*Procyonis lotor*) and in opossums (*Didelphis virgilianus*). A considerable presence of *E. chaffeensis* was reported in the dominant tick species *A. americanum* [13-16, 74]. In Europe, the illness was suspected based upon serological tests in Portugal, in Spain, in Belgium, in the UK [75-77], but this disease has never been proven. The most significant clinical data are undifferentiated fever, accompanied by one or more systemic manifestations: general malaise and cephalea (90%), myalgia, nausea and vomiting (70/80%), arthralgia, diarrhea and abdominal pain; rash is less common than in rickettsioses (20%), interstitial pneumonia and cough (30%), lymphoadenopathy and stupor (15%). Mortality is around 10%. Laboratory data show: leukopenia, thrombocytopenia, anemia, hypertransaminasemia, CRP, elevated ESR, hyperbilirubinemia, hypercreatininemia [78]. Some patients developed a systemic involvement, neurological symptoms or focus deficit. Cases of paralysis of the trochlear nerve and evidence of lymphocytic meningitis [79], and severe myocarditis were reported [80]. Severe infections from *E. chaffeensis* were described in transplanted and immuno-deficient patients [81]. HME causes a serious syndrome in children, state of shock, with kidney failure and respiratory difficulty, cognitive deficit and neurological symptoms [82]. A pediatric case from Venezuela was also recently reported [83]. *E. chaffeensis* may also infect dogs. The infection was first reported in dogs in the USA and, recently, was identified in South America [84].

HGA, *A. phagocytophilum*

The first patient to have recognized HGA was hospitalized in Duluth, Minnesota in 1990. However the infection remained undefined until this new species was detected in 1994, in 12 patients from Minnesota and Wisconsin who had cytoplasmic inclusions (*morulae*) in the leukocytes, and not in the monocytes. There was an immune response reactive to this species and negative for *E. chaffeensis* [67]. This micro-organism was initially, related to *E. phagocytophila* and to *E .equi* [85]. The main vector has been identified as *I. scapularis* in the eastern quadrant of USA and *I. pacificu*s in the west coast. Most cases of HGA have been contracted in geographic regions that are endemic for *Lyme borreliosis* as the host arthropods for *A. phagocytophilum* are also vectors for *Borrelia burgdorferi*. Human anaplasmosis is an acute infection, with incubation period of 5-21 days, occurring prevalently between April and October. Male patients outnumber female patients and about 75% had a tick bite prior to their illness. Differently from rickettsiosis, there is no eschar where inoculation took place. Clinical and laboratory data are similar to those reported for HME but despite clinical similarities each disease may have unique features: a greater severity and higher case-fatality rate for HME, and, in USA, a higher prevalence of opportunistic infections may undergo for HGA. Opportunistic infections are not yet described in Europe where the disease appears generally milder. Older individuals and immune-compromised patients may have a considerably more severe course of the illness. Rashes have been reported only occasionally. Characteristic clusters of bacteria (*morulae*) can be observed in the cytoplasm of peripheral blood granulocytes. Laboratory parameters may show leukopenia, thrombocytopenia, increase in transaminases, CRP and ESR [86]. Acute Anaplasma infection may have a presentation similar to the initial phase of infection by tick-borne encephalitis virus, endemic in Central and northeastern Europe [87] Several cases presented as atypical pneumonitis [88]. One case with fever and facial dyplegia was described in Boston [89]. *E. equi* isolated from cases of equine infection was found to be identical to those isolated from human infection (HGA) in northern California [90]. Seroconversion for *A. phagocytophilum*, testing acute-phase

and convalescent-phase serum samples is a sensitive and specific serologic tool for clinical confirmation; increase in antibody titre remains elevated, even for years. Treatment with doxycycline usually results in complete cure. In Europe, the disease was first described in Slovenia in 1997 [91]. It is not *I. scapularis*, but *I. ricinus* that, in Europe, has the greatest vector responsibility. In Slovenia nucleotide sequences amplified by isolated agents of HGA were shown to be identical to the granulocytic agents isolated from the *I. ricinus* ticks [92]. In Europe serological evidence of human anaplasmosis was reported in most European Countries: in Spain, in the Czech Republic, in Slovenia, in Croatia, in France, Greece, Austria, Poland, Sweden, Denmark, Norway, Finland, The Netherlands [93-105]. Much less cases reported from Slovenia, Spain, Austria, Italy, The Netherlands, Sweden, Poland and France, have been confirmed by presence of morulae in circulating granulocytes, PCR based detection of the organism in the blood and a four fold increase in specific antibody [106-113].
The first case in children, in Slovenia, was described [114]. A case of infection with meningeal consequences, encephalopatia and seizures, was also reported [115]. Interestingly, a case of infection, fulfilling the criteria for confirmed HGA, in a sero-negative patient was observed in Sicily [116]. Serologic testing and genetic identification were also carried out in Sicily on a significant number of animal species, horses, donkeys, cattle, sheep, goats, dogs and guinea pigs. Dogs, sheep and goats were found to be reservoirs for *Anaplasma* [117]. An Italian research reported seropositivity for *A. phagocytophilum* in 6.3% of the human serum examined; 13.3% seropositivity was also found in sheep and 5-6% in dogs. Positivity for *E. equi* was found in horses with and without symptoms. The positivity in human serums, in pets and in stray dogs was confirmed with the identification of *Anaplasma* species circulating in *I. ricinus* [118, 119]. A nosocomial transmission of the disease was described in China [120].

HEE, *E. ewingii*

Several cases of HEE have been linked to *E. ewingii*, an agent previously reported as a cause of granulocytic ehrlichiosis in dogs [68]. *E. ewingii* is the only *Ehrlichia* species known to infect neutrophils. Because the organism has never been cultured, antigens are not available. Thus, infection may be diagnosed by DNA detected in clinical specimens via amplification of specific target by PCR assay. *E. ewingii* has the same vector and vertebrate hosts as HME, The lone star tick, namely *A. americanum*, is the vector of *E. chaffeensis* as well as *E. ewingii*. But *E. ewingii* seems to have a greater spectrum of prevalence in numerous tick species, in particular *D. variabilis* and *R. sanguineus*. The white-tailed deer (*Odocoileus virgianus*) was shown to be host and reservoir also of *E. ewingii* [121]. *E. ewingii* infection may be clinically indistinguishable from infection caused by *E. chaffeensis* and has symptoms and clinical courses similar to HGA: is an acute, febrile illness with leukopenia, thrombocytopenia, anemia, hepatitis, accompanied by headhache, myalgia, arthralgia, vomiting, anorexia. The rash is less frequent than in HME. Severe manifestations include organ (renal and respiratory) failure, pulmonary infiltrates, encephalopathy, meningitis. Most patients with this form of *Ehrlichiosis* had different pathologies causing immune-suppression: transplantation, HIV infection, splenectomy, immunosuppressive drugs [70, 81].

Human infection caused by *E. canis*

The agent of canine monocytic ehrlichiosis (CME), *E. canis*, is an obligate intracellular gram-negative bacterium. Many years have passed since Donatien and Lestoquard described the haemorrhagic illness of dogs, linked to the infestation of ticks and caused by an organism similar to *Rickettsia* [122]. It was not, however, a *Rickettsia*, as believed initially, but an *Ehrlichia*: *E. canis*, genus defined in 1945, a name in honor of P. Ehrlich.
In 1986, a serious febrile illness (intense cephalalgy, myalgia, thrombocytopenia hypoxia, stupor) was described in a 51-year-old patient following a tick bite, complicated by kidney failure, gastrointestinal haemorrhage and systemic candidosis. The case was attributed to *E. canis* but later confirmed as *E. chaffeensis* [123]. The first human infection and culture isolation of *E. canis* was reported in 1996 in Lara, Venezuela, from a 27 years hold veterinarian, apparently chronically infected but asymptomatic. The strain was designated *Venezuelan Human Ehrlichia* (VHE) [124]. The isolated VHE sp., by 16S rRNA base sequence comparison was found to be closely related to *E. canis*. Antigenic and genetic characterization brought to the conclusion that VHE was a new strain or a subspecies of *E. canis* causing asymptomatic persistent infection in humans.
The 16S rRNA 1,408-bp sequence of VHE isolate, subsequently, was demonstrated identical to that of a *Venezuelan Dog Ehrlichia* (VDE) isolated from one dog blood sample and was closely related (99,9%) to that of *E. canis Oklahoma*. An intense circulation of the agent in dogs and ticks, *R. sanguineus*, moreover, was evidenced in the region, all isolates having the same genetic and antigenic profiles. These observations suggested that dog act as reservoir of human *E. canis* infection and that *R. sanguineus* serves as vector [125]. Human infections with *E. canis* in symptomatic patients were also reported from Lara State in Venezuela. Six in a group of 20 patients admitted to the Central Hospital in Lara State, with fever, malaise, nausea, vomiting, diarrhoea, haematological abnormalities were *E. canis* 16S rRNA gene PCR positive. Clinical signs, haematological parameters and age distribution of PCR negative patients were very similar. Patients were young, none of these patients was immuno-suppressed [126]. These observations, again, evidenced the intense circulation of *E. canis* and the fact that infection with this agent may

not be an isolated event in Venezuela. Clinical signs and symptoms caused by human infection with *E. canis* are similar to those presented in HME. Clinical reports vary from asymptomatic infection to severe illness with high-grade fever, headache, myalgia, nausea, vomiting, anorexia. Laboratory findings show thrombocytopenia, leukopenia, liver enzyme abnormalities. Meningitis and encephalitis may occur. Signs are similar in Rocky Mountain spotted fever, dengue fever, mononucleosis or influenza. Differential diagnosis in febrile episodes is important and may led to delay in initiating resolutive therapeutic intervention. From the epidemiological point of view serological cross reaction in *E. canis*, *E. ewingii and E. chaffeensis* infection may brought about incorrect definition of geographic distribution and consequent risk of human infections. Large distribution of the brown dog tick, *R. sanguineus* in other regions of the world, such as the Mediterranean area, were an intense circulation of *E. canis* was evidenced, may led to the suspicion that infection can be misdiagnosed and stress the need for further researches to define the effective risk of infection by *E. canis* in humans.

Laboratory diagnosis

Comprehensive guidelines related to the diagnosis of tick-borne rickettsial diseases (*Rickettsiales Order*) and concerning the epidemiology, clinical approach, treatment and laboratory diagnosis, as already cited, have been provided by experts of CDC in Atlanta, GA, in USA and by the ESCMID Study Group on *Coxiella*, *Anaplasma*, *Rickettsia* and *Bartonella* diseases in Europe [72, 73].

The emerging of new, the high prevalence or the re-emerging of old tick-borne bacterial diseases have intensified the need for diagnostic tests.

Methods in the diagnosis of vector-borne diseases were at the beginnings dependent upon blood smears examination, useful for the detection of *Anaplasma* and *Ehlichia* morulae in circulating blood cells during the acute phase of infection, serological detection of the antibody response by indirect immunofluorescence antibody test (IFA), ELISA or western immune-blotting. *Rickettsiae* were also identified using histological direct immunofluorescence methods on tissue biopsies of the eschar when present [127, 128]. Two or more serum samples, collected during the acute phase of infection and after two or three weeks, during the convalescent phase, are needed for serology. IFA test is the reference test used for antibody detection [129]. Different strain are used in different research centres as test antigens. Intracellular grown or cell-free antigen prepared by purification of infected cell culture, coated on glass slide and fixed with acetone or alcohol, are used for testing. Serological confirmation of infection requires seroconversion or at least a ≥ 4 fold increase in antibody titre. Immuno-reactive proteins of the external membrane from *E. canis* were recently used to monitor the antibody response and to attempt to develop specific ELISA [130, 131].

Isolation of germs from tissue and blood, in tissue cultures or in embryonic eggs, is difficult and requires laboratory equipments and trained personnel. Isolation of the agent requires blood samples collected during the acute phase of the infection. Although *Anaplasma* was able to resist for many days in samples kept at room temperature, blood samples must be kept at room temperature or at 4°C for not more than two days and frozen at –20°C if is needed to maintain infectivity for longer periods. Blood must be collected on EDTA, as heparinised samples may compromise results of PCR based methods. Blood smears for the observation of morulae in WBC must be prepared as soon as the blood is taken, air-dried and kept at room temperature. Cultivation need a level 3 biohazard laboratory. Cells, promyelocytic HL-60 leukemia cell line, are commonly used, maintained in RPMI-1640, antibiotic free medium, supplemented with glutamine and fetal bovine serum. 25 cm^2 flasks containing medium with 2 x 10^5 cells/ml are inoculated with 100 μl of fresh blood or 0,5 ml of the leukocyte fraction of the frozen blood. Using Giemsa staining, morulae can be observed on days 3-7 [132, 133].

The development of DNA based approaches enhanced sensitivity, specificity and rapidity of a possible diagnosis, increased knowledge in epidemiological information, provided support to the clinicians for a better understanding of distribution and differences in the clinical features of these diseases. Molecular approaches have included PCR sequencing, RFLP analysis of amplified genes: the 16S rRNA gene, the 16S-23S rRNA intergenic transcribed spacer (ITS) region and many others. PCR assays targeting fragments of the msp2 homologues or the 16S rRNA gene seemed to be the most sensitive. To confirm identity, however, sequencing of the PCR products is necessary [134-136].

Order rickettsiales

Improvements in molecular methods led to a modification of the *Rickettsiales Order*, which has recently undergone an important reorganization [137]. The *Order*, therefore, contains three families:

- Family *Rickettsiaceae*;
- Family *Anaplasmataceae*;
- Family *Bartonellaceae*.

From the *Family Rickettsiaceae* have been removed numerous elements. The *Rickettsia* genus has maintained the two "classic" groups of *Spotted Fevers* and *Typhus*. For the *Rickettsiae* of the *Scrub Typhus group*, which are different because do not have a peptoglican and lack an external capsule-like layer, a new genus has been created: *Orientia* (*Orientia tsutsugamushi*) [138].

All the members of the *Ehrlichia*, *Neorickettsia* and *Wolbachia* have been transferred to the *Anaplasmataceae* family. The reorganization of the genera within the family *Anaplasmataceae* was based on the comparison of sequences from rrs and groESL operon and on the complete sequencing of several species of the family.

Genetic analyses in this family, in fact, have identified four distinct clusters:

- *Anaplasma*;
- *Ehrlichia*;
- *Neorickettsia*;
- *Wolbachia* [139] (Fig. 1).

The *Anaplasma genus* of the *Anaplasmataceae* family now includes several species. Three species infect erytrocytes: *Anaplasma marginale* (*A. marginale*), responsible of a severe febrile haemolytic anemia in cattle. *Dermacentor andersoni* and *Boophilus microplus* are the vectors. *Anaplasma centrale* (*A. centrale*) produces the same disease, usually mild in cattle, *Anaplasma ovis* a pathogen of sheep and goats. Two species infect WBC: *Anaplasma bovis*. the agent of bovine anaplasmosis, transmitted by *Rhipicephalus appendiculatus* and *Amblyomma variegatum*, infects monocytes, and *A. phagocytophilum* (ex.: *E. equi*, HGA) has as targets granulocytic cells. *Anaplasma platys* the agent of cyclic thrombocytopenia in dogs, transmitted by *R. sanguineus*, infects platelets. Some genetic differences between *A. marginale* and *A. centrale*, that tend to cluster together and *A. phagocytophilum*, have suggested that these species may be a distinct genus. It was, in fact, proposed to separate *A. phagocytophilum* from the group and, eventually, return it to the genus *Cytoecetes* as *Cytoecetes phagocytophila*.

The *Ehrlichia* genus includes several human pathogens [140]: *E. canis*, *E. chaffeensis*, parasite circulating monocytes of humans and animals; *E. ewingii* infects granulocytes of dogs and man. *Ehrlichia ruminantium* (*E. ruminantium*), formerly *Cowdria ruminantium*, transferred to the genus *Ehrlichia* [141], where it forms a distinct clade, is the agent of a severe disease of ruminants, the *heartwater*, infects endothelial cells and, beside the heart (*hydropericardium*: *heartwater*), may causes encephalitis. DNA of *E. ruminantium* has been detected in young patients [142]. *Ehrlichia muris* (*E. muris*), which also forms a distinct clade in the genus, is transmitted by the tick *Haemaphysalis flava* and infects rodents. *E. muris* has not been identified, yet, as agent of human disease.

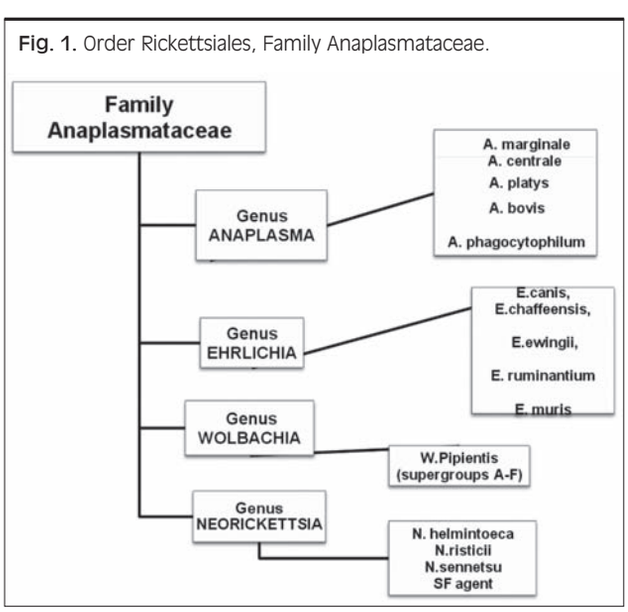

Fig. 1. Order Rickettsiales, Family Anaplasmataceae.

Perpetuation of the genus *Neorickettsia*, formerly *Ehrlichia* spp, depends on trematodes and not on ticks. *Neorickettsia helmintoeca* infects the salmonid fluke *Nanophyetus salmincola* and causes a febrile illness in bears and dogs. The Potomac horse fever, a febrile illness with intestinal troubles and diarrhoea in horses that have ingested aquatic insects contaminated with metacercaria, is caused by *Neorickettsia risticii*. Japanese and Malaysian residents that eat uncooked fish possibly contaminated by some kind of flukes infected with *Neorickettsia sennetsu* may develop a mononucleosis-like disease. The fluke *Stellantchasmus falcatus* hosts the SF agent, a *Neorickettsia* which has not been reported in mammalian infective events.

Wolbachia are intracellular bacteria found in arthropods and in filarial nematodes [143, 144]. *Wolbachia persica*, formerly included in the genus, has been reclassified as *Francisella persica*. *Wolbachia melophagi* has an undetermined taxonomic position and seems more like the gammaproteobacteria [145].

Wolbachia pipientis, the sole species of the genus, has a complex molecular diversity and, by polyphasic gene analysis, six main different groups have been identified, named, at the moment, *super-groups A-F* [146]. Genetic divergences among the different groups may suggest that each group may be a single species with different variants. The single name, however, is maintained until new data may better clarified taxonomy and phylogeny [147].

The family *Bartonellaceae* are important zoonotic pathogens. The number of known *Bartonella* spp is rapidly increasing; intermediate hosts are much more, in quantity and diversity, than previously thought, vectors are as well numerous and most of them not fully defined [148]. Various domestic and wild animals can serve as chronically infected reservoir hosts for various B. spp. Transmission is due to cat fleas but other vectors, ticks and biting flies, have been identified. *Rochalimea* has been included in the *Bartonellaceae* family. Cats can be infected with *Bartonella henselae* (*B. henselae*), *Bartonella clarridgeiae* (*B. clarridgeiae*), *Bartonella bovis*, *Bartonella koehlerae* (*B. koehlerae*), *Bartonella quintana* (*B. quintana*). Cats are an important reservoir and as many other species which act as reservoirs usually display chronic bacteriemia. Dogs can be infected by *Bartonella vinsonii* (*B. vinsonii*), *B. henselae*, *B. clarridgeiae*, *Bartonella washoensis*, *Bartonella elizabethae* (*B. elizabethae*), *B. quintana*. Dogs develop a disease spectrum similar to humans and may be sentinels for infections in humans and a good research model. 7 of 19 *Bartonella* species are potential pathogens for humans. *B. quintana* and *B. henselae* agents, respectively, of the trench fever and CSD, may also cause endocarditis and bacillary angiomatosis in immunodepressed patients. *Bartonella bacilliformis* is the agent of Carrion's disease. *B. elizabethae* and *B. vinsonii berkhoffii*

cause endocarditis, *Bartonella grahamii* (*B. grahamii*) neuroretinitis, *B. vinsonii arupensis* may affects the heart valves [149, 150]. Genetic analysis identified, at the moment, six evolutionary clusters. *B. henselae*, *B. koelherae* and *B. quintana* cluster togheter, as well as *B. vinsonii vinsonii* and *B. vinsoni berkhoffii*.

Bartonella bacilliformis and *B. clarridgeiae* seem to be divergent species. A cluster includes *Bartonella tribocorum*, *B. elizabethae*, *B. grahamii* and a group of strains associated with rodents indigenous of the Old World and an other one includes bacteria isolated from various rodents belonging to native species of the New World [151, 152].

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