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Past, Present and Futur of Rickettsial Diseases in Montenegro

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SUMARY

Rickecttsioses have played a significant role in the history of world civilization. (1,2,3) Epidemic typhus has affected over 30 million people in both world wars, with the outcome of 3 million people who died. Additionally, during World Wars I and II, it hit all Balkan countries hard. (4). The tragic outcome of the epidemic in Serbia and Montenegro during World War I, resulted with over 100,000 victims and more than half of the entire medical staff infected, entered the history of medicine. (5,6,7) In the XX and XXI century, the renaissance of rickettsial diseases is characterized by the reappearance of typhus in the world with high mortality and the appearance in regions of the world where it did not exist previously. The drastic increase in rickettsial spotted fever (abb. SFRD) in the late 1970s, ehrliosis in 1990, testify to the strong association of these diseases with changes in the eco-environment, which were largely influenced by human activity. In addition, there are difficulties for health systems to predict epidemics of these diseases and protect the population at risk. (8,9,10)

Drastic changes in the eco-environment have contributed to increasing adaptability of rickettsial agents, as well as the possibility of acquiring the new features. They influenced changes in vector properties and their expansion, contributing to the return of old and the appearance of new rickettsioses in the Vector Borne Diseases (abb. VBD) group, emphasizing Hans Zinzer's prediction: "Typhus is not dead, moreover it exists in the nature and will always return, as long as human stupidity allows it."(2,3)

A retrospective - prospective study of rickettsial diseases in Montenegro conveyed until the end of 2017, used historical data for epidemic typhus. For other rickettsioses, in the period from 1996 to 2017 standard epidemiological, clinical and laboratory methods were used. For the etiological confirmation of the clinical diagnosis of rickettsioses, and the detection of multi etiological (co-

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infectious) forms of the disease, serological tests were used: Indirect immuneo fluorescence (IIF), ELISA, and latex agglutination test, for detection of specific antibodies. From the group of micromolecular methods, the results of Polimerase Chain Reaction (PCR) for DNA detection, were of special importance. (7)

Montenegro is an endemic area for numerous rickettsial diseases. In our country, endemic-epidemic typhus has not been registered since 1946. In the period from 1996 to 2017, we analyzed 657 patients with an etiologically confirmed diagnosis of various rickettsial and rickettsial-like diseases.

The first serological confirmations of rickettsial spotted fever (SFRD) and Q fever were registered in 1995/1996. By the end of 2017, 293 cases of rickettsial spotted fever and 158 cases of Q fever were registered. The first serological evidence of ehrlichiosis was obtained in the period from 2008 to 2017; with serological confirmation of Ehrlichiaecanis in 64 cases. Bartonelosis was confirmed in 42 cases in these studies.

Clinically, rickettsial diseases vary greatly in relation to severity, clinical presentation, from self-limiting to fulminant, life-threatening diseases. The problem of modern presentation of these diseases is evidenced by their increasingly frequent registration in co-infectious forms with the participation of various rickettsial agents, wider range of agents of VBD complex, as well as agents that do not belong to this complex, which was proven during our tests. The multi etiological (co-infectious) form of the disease additionally complicates the diagnosis, therapy and prognosis of the disease. It affects the creation of new species and subspecies of rickettsial agents, which move from enzotic cycles to zoonotic cycles, without the need for long-term evolution in enzotic cycles.

From ancient times to the present day, rickettsial diseases represent a significant public health problem in relation to diagnosis, therapy and prognosis, with possible negative implications for the future.

Keywords: Rickettsioses, emerging diseases, past, present, future

INTRODUCTION

Rickettsioses are a group of diseases classified as zoonoses. They are represented by varies of rickettsial syndromes and similar diseases. Rickettsia (R) is a gram-negative protobacteria, coco bacillus obligatory intracellularly, that parasites in the nucleus or cytoplasm of eukaryotic cells. (11,12). Long before the discovery of the etiological basis of the disease, the connection between these diseases and arthropods was suspected. They were later classified as Vector Borne Diseases (VBD). (13). The remarkable ability to adapt, and the consequent change of properties of these zoonotic agents, have enabled their transmission via arthropod vectors, as well as in different other ways (C. burnetti). (14-17). The etiological basis of the disease has been known since 1987, when gram-negative cocobacilli were isolated from infected human tissues. Subsequently, these agents were isolated from tick tissue, without visible cellular damage. On

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the basis of this finding, Wolbach hypothesized that "over billions of years, there must have been a symbiotic evolutionary link between these microorganisms and arthropods." (1)

These ancient microorganisms survive in the cells of natural mammalian hosts and arthropod vectors for thousands of years (18,19). They are phylogenetically classified between bacteria and viruses. The diversity of rickettsiae is due to different specific sites in the cells in which they survive. Furthermore, it is also due to significant differences in their major outer cell membrane proteins, as well as genetic linkage. (18) The example of extreme adaptability of C. burnet ti is manifested through increased metabolic activity in acidicenvoirment of phagolisosomes. That envoirment is not suitable for survival of many other microorganisms (14).

Some microorganisms from the family Rickettsiaceae are genetically closely related to each other (e.g. R rickettsii, R akari, R prowazekii and R typhi); others are less associated (e.g., Ehrlichia and Bartonella), and some are not associated with rickettsiae (e.g.C burnetii). The phenotypic characteristics of the clinically important microorganism Orientia (Rickettsia) tsutsugamushi suggest that the species may be an example of convergent evolution in similar ecological conditions of endemic foci. (20,21) The prevalence of rickettsiae in the nature, depends on the number and species of natural hosts, number and species of infected arthropods, which for most species of rickettsial agents they have a dual role, natural hosts and vectors**.** (19).

Significant qualitative and quantitative changes in the epidemiological and immunological characteristics of rickettsial agents in natural cycles, with the expansion of the spectrum of vectors and changes in their affinity for the hosts on which they feed, ensure their expansion throughout the world. The discovery of competitive relationships among rickettsial agents, and with other agents of the VBD complex (borrelia, rickettsiae, ehrlichiae, babesiae), and others (e.g. brucellosis), in primary hosts, arthropods and human infections/co-infections, are characteristics of modern traits, which are reflected in the new characteristics of rickettsial diseases. (22, 23)

All rickettsial infections begin with the intrusion of microorganisms through the skin, during the blood meal of hematophagous vectors (ticks, fleas, lice, mites), or by rubbing the feces of the vector through small abrasions on the skin.

Adherence to host cells is the first step in pathogenesis. (24). Rickettsiae induce activation of host phagocytic cells. In the second step, they multiply in the cytoplasm or nucleus of infected endothelial cells and the muscular layer of the microcirculation at the site of skin penetration, with the formation of intracytoplasmic inclusions and vasculitis, with perivascular mononuclear infiltrates, consequent necrosis of endothelial cells, necrosis of endothelial cells, thrombosis and ischemia. (25)Non-efficacy of the first lines of immunological defense and the prevailing of infection are confirmed by the findings that non-opsonized rickettsiae phagocytosed by inactivated macrophages survive and multiply in phagocytic cells. (25). Additional studies have shown that in an infected organism, rickettsiae close in the corresponding mitochondrial areas, where they significantly reduce their genomic structure, necessary for maintaining metabolic and structural biosynthesis. (26-28). One of the first clinical signs of non-effective inflamation

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response of the host are enlarged regional lymph nodes. Hematogenous dissemination of rickettsial agents expands the spectrum of clinical manifestations.
Rickettsioses vary greatly in severity, but regardless of the subclinical or clinically manifest course, they are generally considered to be the cause of severe and dangerous human diseases (29 - 33). Modern research indicates that rickettsial diseases are an increasing and significant public health problem in the world. As a rule, co-infectious forms of the disease have an altered and more severe clinical course. They further contribute to diagnostic difficulties, problems of uncertain effect of therapeutic treatment and uncertain prognosis of the disease. (6)

Diagnosis and isolation of rickettsial agents is very difficult, as well as dangerous (8,34). They cannot be grown on artificial feeders, but only in living cells. Their isolation and identification is performed in specialized laboratories, which have modern equipment and methods, and which can identify rickettsiae immuno histologically from skin biopsies. (35) For diagnosis in routine practice, serological methods IIF, ELISA, latex agglutination are used to detect specific antibodies. Another group of modern methods relies on PCR and other micromolecular methods, and evidence of the presence of rickettsial DNA. The results of molecular research also introduced changes in the taxonomy of rickettsial agents. They are classified into genera and bio-groups(36-43).
Although early treatment with doxycycline, tetracyclines, or chloramphenicol has generally been shown to be effective, in some cases, efficacy is lacking. (44-48)
Preventive, epidemiological measures are of particular importance for the protection of people. (49). They include monitoring of vectors and reservoirs, use of insecticides and early removal of ticks from the skin, as well as control of rodent populations (rats, mice) and prevention of their access to households, domestic animals, pets. Vaccines against epidemic typhus and typhoid rickettsiae have been developed empirically. Dead vaccines did not provide complete protection. (50). Live attenuated vaccine against epidemic typhus has been shown to be effective, but with a significant frequency of side effects. (51). For Q fever, there is also a vaccine, which is used to protect animals and exposed categories of people, but its effect is also insufficiently understood. (52). The presence of strong immunity in reconvalescent subjects indicates that the development of vaccines is feasible, but requires serious studies of rickettsial antigens and an effective natural anti-rickettsial immune response of the host, e.g.T-lymphocyte-mediated immune mechanisms, including the effects of lymphokines, tumor necrosis factor (TNF-gamma), interleukin-1, and others. (31).
In the past, rickettsioses have been a major and significant public health problem in relation to diagnosis, therapy, and prognosis. In the 21st century, in addition to the progress of science, technology, therapeutic and preventive possibilities, they are experiencing their renaissance. Due to the return of the old and the appearance of new dangerous rickettsioses, they are still a significant public health problem, with expected negative implications in relation to the future.
Since 1984, "new" pathogenic species have been isolated and identified, especially in the group of rickettsial spotted fevers, so that today 28 serotypes and numerous new subspecies are known. Besides pathogenic rickettsiae and those presumed to be pathogenic to humans, numerous tick-borne rickettsiae have been isolated and identified, whose pathogenic potentials are unknown at this time, with expectations that the spectrum of pathogenic human rickettsiae may continue to

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increase in the future. (53). According to the newer classifications, rickettsiae are classified into groups on the basis of serological and molecular methods. Additionally, on the basis of differences in antigenic structure and biological characteristics of the species in the genus rickettsiae, they are classified into biotypes (54): R. spotted fever, R. spotted typhus, R.archaic (R.bellii and R.canadiensis (found only in ticks and their pathogenicity to humans has not been confirmed). Classifying rickettsias in the gropus is conducted based on the differences in antigenic structure and biologic properties of the species in the genus of ricetssias. (Figure 1)

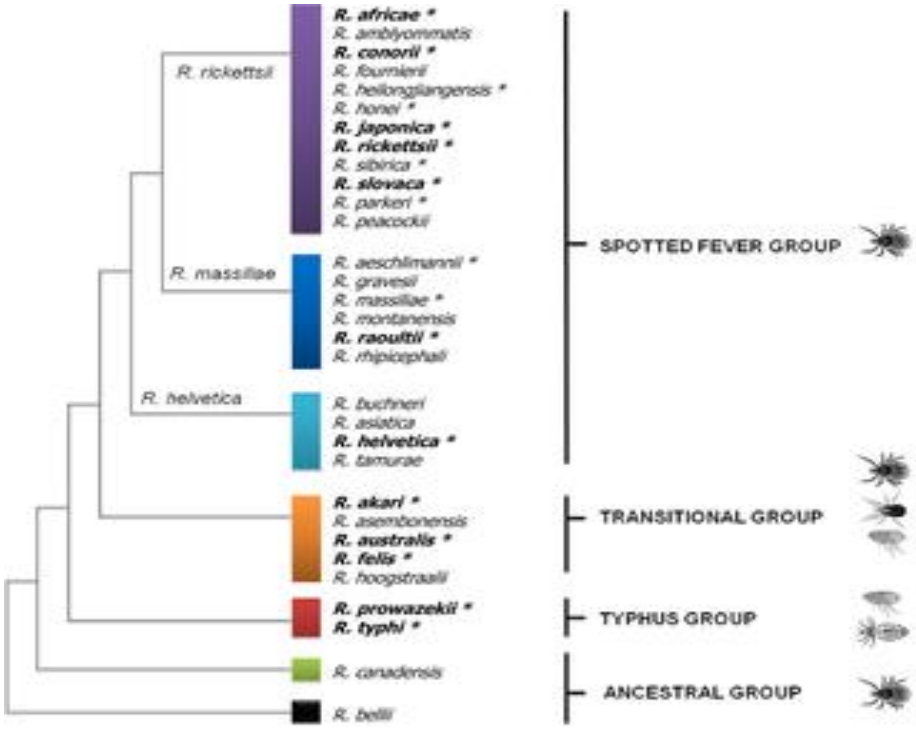

Figure 1. ***Classification of rickettsial agents into biogroups***

Typhus biogroup: Includes 2 entities. Classical endemic - epidemic typhus and relapsing typhus (Brill Zinzer disease). The causative agent is R. prowazeckii. Another member of this biogroup is rat typhus. The causative agent is R. typhi (formerly moserii). It causes a disease, clinically similar to rickettsial spotted fever, but antigenically different and, as a rule, considerably more severe. (55).

Scrub typhus biogroup (abb. SFG) is caused by Orientiatsutsugamushi. It is transmitted through the larvae of thrombiculed mites, causing Tsutsugamushi fever (scrub typhus or shingles). This group contains three main serotypes: Kaep, Gilliami, Kato. (56)

Biogroup of spotted fevers. Of the many 28 species Previously encountered under different names: R. rickettsii, the causative agent of rocky mountain sppoted fever (RMSF), R. acarii, the causative agent of rickettsial smallpox, R. conorii the causative agent of Mediterranean spotted

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fever, which is found in the medical literature under different names: Boutoneous fever of Tunisia, Marseille fever, Kenyan fever, African typhus, Israeli spotted fever, Indian tick typhus. (Table 1). (57)

Transitional (archaic) biogroup. In this group are the genera: R. acarii, the causative agent of rickettsialpox; R. felis, the causative agent of Flea borne spotted fever. They are genealogically related to R. belli and R. canadiensis. (58)

Other rickettsioses and closely related illnesses:
In the last ten years, new rickettsioses have been described, including lymphadenopathy with necrotic echara (Tibola) or tick-borne Dermacentormarginatus (Debonel). The causative agents are R.slovaka and R.raoultii (59).

The genus previously named Rochalimae is classified in the family Bartonellaceae. It is related to Trench fever (60). According to a new, recent classification, the species Bartonela, Coxielae no longer belong to the order Rickettsiales.

In the last 30 years, many new rickettsiae in ticks from different areas of the world have been found using molecular techniques. Some of these rickettsiae are: R. slovaca, R. aeschlimannii and R. Rhipicephali, and they were found in the former Yugoslavia (in Croatia). R. slovaca, R. helvetica, R. aeschlimannii, R. raoultii were found in different parts of the world (Table 1). Historical data, our and veterinary tests have shown that Montenegro is an endemic area for many rickettsial and related diseases.

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Table 1. Spotted fever including Rocky Montain Spotted fever and Mediteranean spotted fever	
Organism/Disease	Distribution
R.aechlimani	Southern Europe, Africa, Organism also faund in Ticks in Kazasthan
R.africae (African tick bite fever)	Africa and the eastern Caribean
R.acarii (Ricketsial pox)	Organism is cosmopolitan, including North America, Europe, Asia, Mexico, Africa. Disease reported exspecialy in urban areas of northeastern US, but also in Europe (Croatia, Ukraine and Turkey), Korea and other areas.
R.australis (Quisled tick typhus)	Australia
R.conoriissp.conorii (Mediteranean spotted fever)	Mediteranean: also foci in northern and central Europe, Cases reported in Subsaharan Africa
R.conoriissp.indica (Indian tick typhus)	Indian subcontinent
R.conoriissp.caspia (Astrakhan spotted fever)	Russia (especially the Astrakhan region on the Caspian Sea), Kazakhstan, one case in Chad. Organism also found in tick in Kosovo
R.conirii ssp. israelensis (Israeli Spotted fever)	Midle East, Portugal. Organism also found in tick in Italy (Sicily)
R.felis (Flea- borne spotted fever)	Possibly worldwide
R.heilongjangiensis (Far Estern tick borne rickettsiosis)	Russian Far East. Organism also found in tick in China, possibility associated with human diseases.
R.helvetica	Europe, Japan, Thailand, Laos
R.honei (Flinders Island Spotted Fever)	Australia, Nepal, Organism also found in tick in Thailand, Sri Lanka, Italy, US (Texas)
R.japonica (Japanese spotted fever)	Japan and South Korea, possibly other nearby regions
R. massiliae	Europe, Africa,(Emerging disease in Mediteranean region). Organism also found in ticks in the US and South America
R. monacensis	Spain, Italy
R. parkeri	US, Uruguay, Organism also found in ticks in Argentina, Brasil, Uruguay
R. raoultii (TIBOLA / DEBONEL)	Europe, Russia, Organism also found in China
R. rickettsii	Western Canada, Continental US, Mexico, Panama,

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(RokyMontainSppoted fever)	Argentina, Brasil, Bolivia, Colombia, Costarica
R.sibirica ssp. mongolitymonae (lymphangitis associated rickettsiosis)	Africa, Europe (France, Greece), China
R. sibiricassp.sibirica (Siberian tick typhus)	Northern Azia, including part of Russia and China
R.slovaca (TIBOLA/DEBONEL)	Europe, Russia, Organism also found in tick in China. R slovaca is associated with two tick species that are common from Europe and Asia

METHODOLOGY

Retrospective - prospective clinical research of rickettsial diseases in Montenegro, until the end of 2017, was undertaken on the basis of historical data that Montenegro is classified as an endemic area for endemic epidemic typhus, which has not been registered since 1946.

Rickettsia and related diseases, which are present and current in Montenegro today: Rickettsial spotted fever (SFRD), Q fever, Ehrlichiosis, Bartonellosis, as independent entities and in co-infectious forms, were analyzed in the period from 1996 to 2017. Tests were conducted at the University Clinic for Infectious Diseases in Podgorica. The analysis included 657 respondents. Serological methods IIF, ELISA, Western blood test were used for etiological confirmation of clinical diagnosis. Micromolecular diagnostics relied on the results of the PCR method. For the diagnosis, differential diagnosis and detection of multi etiological forms of rickettsial diseases with a wide range of agents of the VBD complex, additional serological methods and other micromolecular methods, biochemical, X-ray methods, ECG, ultrasound diagnostics, etc. were used, if necessary.

RESULTS

In the period from 1996 to 2017, the analysis included 657 subjects with etiologically confirmed diagnoses of various rickettsial and rickettsial - like diseases. The first serological confirmations of rickettsial spotted fever (abb. SFRD) and Q fever in Montenegro were registered in 1995/1996. By the end of 2017, 293 respondents with SFRD and the causative agent R.conorii were registered. In 75% of the patients in addition to the basic agent, R.typhi and R.acarii have been registered as co-infecting agents. Q fever was diagnosed in a total of 158 cases. The first serological confirmations of ehrlichiosis were obtained during 2008/2017,by serological confirmation of Ehrlichiaecanis in 64 subjects, presented as human granulocytic ehrlichiosis (HGE). Bartonellosis, or Bartonella henselae, was first confirmed in 2007, first with a clinical picture of cat scratch disease (abb. CSD), and later with a wider range of clinical manifestations of this disease, in 42 cases. (Table 2)

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Table 2. SEROLOGICAL DIAGNOSIS OF RICKETTSIAL DISEASES IN MONTENEGRO		
Rickettsial diseases	**Hystory, Confirmation, Methodology**	**Registration 1996 - 2013**
Typhus exanthematicus -R.tiphy (mossery)*	Balkans and I, II World Wars VMA Belgrade (IIF and PCR)	After1945 is not registered 94+
Spotted fever rickettsioses -R.conorii -R.acarii*	-First serological verification **1996** -Method (IIF: 1: 1450) -VMA, Belgrade and Vet.laboratory, Podgorica	293 76+
Q fever -Coxiellaburneti	-First serological verification **1995/1996** -Method: IIF, Elisa, WB	158
Ehrlichiosis -Ecanis	First serological confirmation **2008** -Method: IIF, Elisa, PCR -Veterinary laboratory, Podgorica	64
Bartonellosis (CSD) -B. hens ellae	First serological confirmation **2008** -Method: IIF, Elisa, PCR -VMA Belgrade Institute for Public Health of Montenegro	42
Total		657

*+R.tiphy-94 cases and *R.acarii-76 cases are registered just serologically, in co-infections with R. conorii*

Throughout the history of the human race, epidemic typhus has been classified as an obligatory companion of misery, wars, and natural disasters. Serbia and Montenegro were the last countries in Europe to report cases of spotted fever, Montenegro in 1946 and Serbia in 1948. Since then, it has not been registered in our country.

Epidemic typhus, belongs to endemic VBD. Brill Zinsser's disease is a recurrence of epidemic typhus. After an acute infection, R. prowazecki can persist for years in the lymphatic tissue of a person in the dormant stage. After a long period of latency, it can be reactivated and manifested as recurrent epidemic typhus, which clinically resemble the primary one, but it is usually milder and last shorter. Exanthema on the skin is rare.

During World War I, epidemic typhus hit all the Balkan countries and the Eastern Front hard. The tragic balance in Serbia and Montenegro, with over 100,000 victims and more than half of the entire medical staff infected, has entered the history of medicine. This epidemic of epidemic typhus in 1914/15 in our area was the most rapid emerging epidemic, the fastest in spread, the largest in strength and the fastest stopped of all epidemics in history (1,2,6,7). It was specific as the first major epidemic of this disease, which was stopped by "dissecting". Hence, this

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suppression is prototypical, because there was no role model to follow and their experience to apply. (Figure 2).

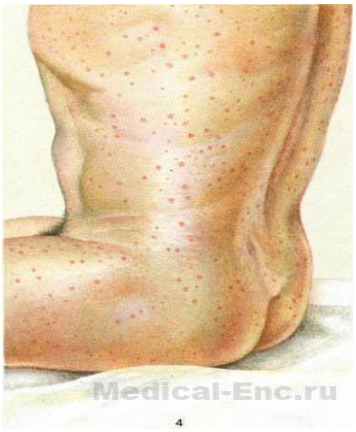

Figure 2. ***Rezervoari, sisari : životinje (glodari), čovjek. Inkubacija 10 – 14 dana, traje 13 – 14 dana. Izraženopštiinfektivnisindrom. Egzantem: najprije u aksilama, po koži trupa, odakle se širi ka periferiji. Rijetko zahvata lice, dlanove i tabane. Između 5. – 12. dana u serumu oboljelih javljaju se aglutinini prema proteus OX-19 (Ređe OX-2), što se dokazuje Weil Felix-ovim testom (WFT).***

Many unknown facts follow this severe and highly deadly disease up to this day, ranging from enigmatic interepidemic maintenance to enigmatic behavior in an infected organism. R. prowazekii infects endothelial cells of blood vessels with consequent disseminated vasculitis. Unlike the SFRD group, epidemic typhus occurs in winter. It is transmitted by the physical (white) louse (pl. lice). The problem of its interepidemic maintenance has been demystified in the United States by isolating R. prowazecki from Southern flying squirrels and their ectoparasites, including the flea Orchopeashoward and louseNeohaemtopicusscripotera, which are the causative vectors. Body louse and the person suffering from Brill Zinsser's disease, in peacetime conditions, are not of great importance for the inter-epidemic maintenance of epidemic typhus. (7).

Given the endemic nature of the disease, it is quite certain that some other species of rodents are natural reservoirs of this pathogen, but so far in this direction, no tests have been performed in our environment. Since 1997, reactivation of spotted fever in the world, with high mortality and occurrence in regions of the world where it did not exist, warns that typhus is not dead (Hans Zinsser).

The problem of the survival of the causative agent in the human body after the infection has also not been clarified. Recent studies of R. prowazecki have shown that it probably originates from eukaryotic mitochondria. It turned out that the complete sequence of genes of this protobacterium of 1,111,523 base pairs contains 834 genes that encode proteins. The functional profiles of these genes show similarities with mitochondrial genes. There are no genes required for glycolysis in either the R. prowazekii genes or in the mitochondria, but the complete set of

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genes encoding the tricarboxylic acid cycle components and respiratory chain complexes is found in both. The production of ATP in rickettsiae is equal to that in mitochondria. Many genes involved in the biosynthesis and regulation of amino acid and nucleoside biosynthesis in free-living bacteria are absent in R. prowazekii and mitochondria. It is possible that such genes have been replaced by homologues in the nuclear (host) genome.

Phylogenetic analyzes show that R. prowazekii is closer to mitochondria than to any bacterium on the Tree of Life.

Murine typhus: Another member of the typhus biogroup is R.typhi (formerly Mosserii). Natural reservoirs are numerous species of mammals, rats and rat fleas (in which the transtadial and transovanial passage of this rickettsiae has been registered). The vectors are rat fleas and ticks. Incubation is 1 - 2 weeks after the injection. The main symptom is the appearance of exanthema, which does not differ from that of the SFRD group, but does not occur in all patients. (Figure 4). R.typhii is a frequent participant in coinfections (75%) with rickettsiae of the SFRD group (R. conorii, R.acarii), which further complicates the clinical diagnosis of this participant in coinfections. (Figure 3)

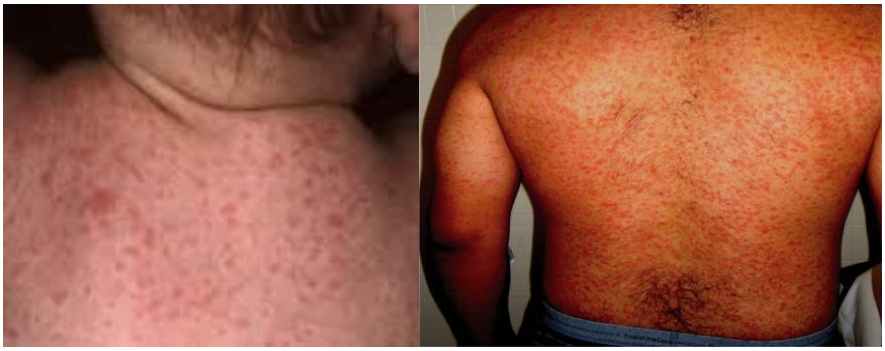

Figure 3. ***In 75 % of patients with SFRD, R.tiphy and R.acariare registered as co-infectious agents along with R. conorii.***

Spotted Fever Rickettsiosis (abb. SFRD)

They were first described in the Mediterranean area in 1910 and on the American continent in 1891. Endemic distribution around the world has caused different local and regional names for this group of rickettsiae. (Rocky Mountain spotted fever in North America, Queensland tick spotter in Australia, Boutoneous fever in North Africa, Kenyan tick typhus, Marseille or Mediterranean fever, etc.). The first serological confirmations of SFRD and Q fever in Montenegro were registered in 1995/1996. By the end of 2017, 293 cases of SFRD were confirmed and the causative agent of R. conorii had been registered. All rickettsiae from this group cause similar diseases, which after incubation for 7-10 days, begin abruptly with pronounced symptoms of general infectious syndrome. After 3-5 days generalized maculopapular rash appears, which begins from peripheral parts of the body (palms, feet) and progressively spreads centripetally. As the disease progresses, lesions of the central nervous system, cardiovasuclar system, and other systemic disorders become evident. These diseases

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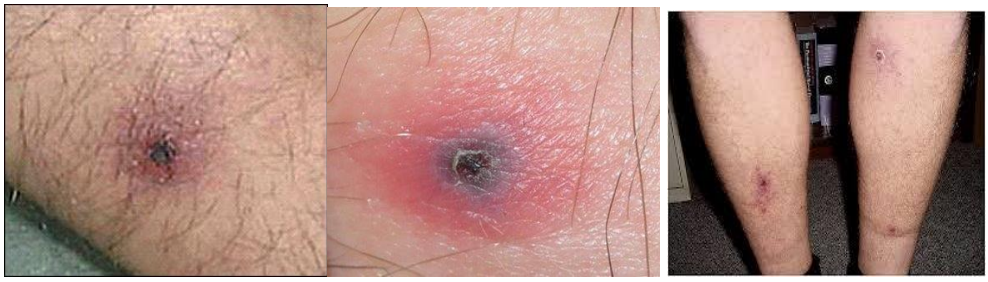
were first classified as short-lived febrile, exanthematic diseases, mostly with a good prognosis. However, practice has shown that in most cases, they do not deviate in severity and are difficult to differentiate clinically from typhus exanthemicus. Systemic disorders were significantly more severe and diverse in patients with a multi etiological basis of the disease. (Figure 4, 5)



A B C

Figure 4. ***The first diagnosed cases od Mediterranean spotted fever, primarly recognized on the basis of anamnestic information about tick bite. A, B - Echar on the site of tick bite, C- multiple tick bites and echar (Original photo documentation by Prof dr.B. Andrić)***

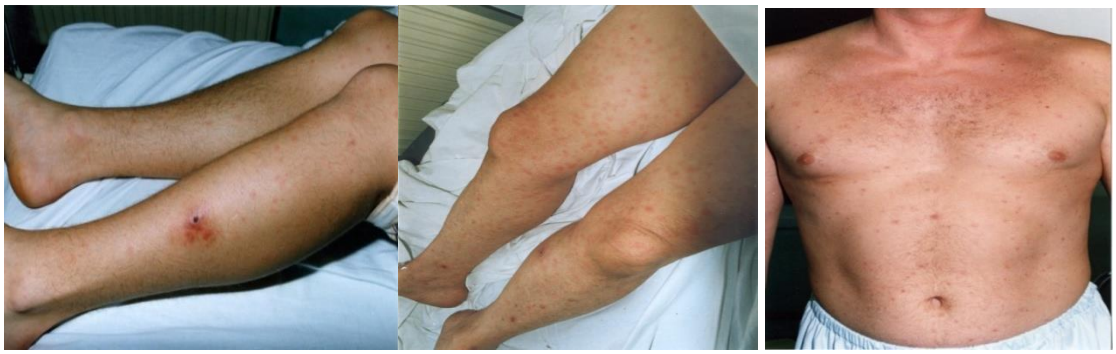


Figure 5. ***Mediterranean spotted fever. Maculopapulous rash, which is spreading centripetally from the perifery (Original photo documentation by Prof dr.B. Andrić)***

R.acari (Rickettsial pox)

This rickettsiosis in Montenegro is not clinically recognized, but using serological and IIF methods, results were obtained in which with a clinically clear picture of Mediterranean spotted fever, serologically diagnosed specific antibodies to R.acari and R. typhi in 75% of subjects. By additional diagnostic methods (PCR) and monitoring of the titer of specific antibodies by the IIF method, it was confirmed that this is a multi etiological (co-infectious) form of the disease. The natural reservoirs for R.acari are mice and mouse fleas, which are also the basic vectors. Transovarian transmission of rickettsiae to offspring has been demonstrated in fleas. After incubation for 7 - 10 days, in the first stage, at the site of the flea bite, papule/papules first develop, which quickly exulcerate, forming a primary rash (primo affect). The second stage is characterized by a sudden onset, with pronounced symptoms of the general infectious syndrome,

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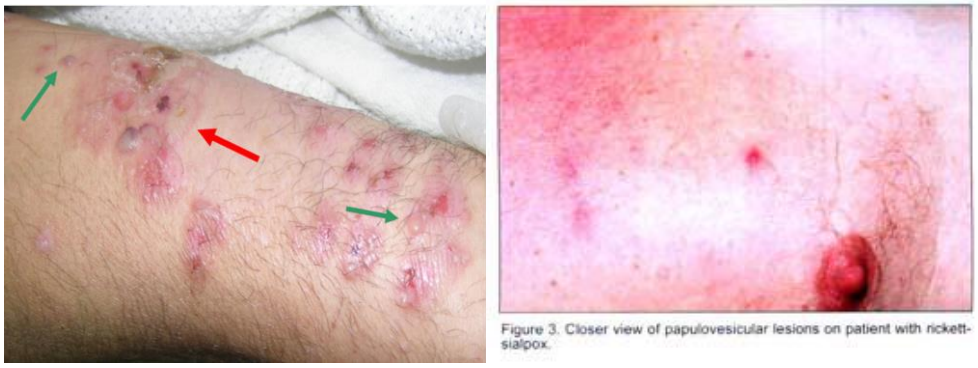
and the appearance of generalized exanthema, which is papulovesicular and lasts for a long time. The prognosis is generally good.(Figure 6)



A B

Figure 6. ***Rickettsialpox. During our examinations this ricketssiosis was not clinically recognized, but serological test discovered this ricketssiosis in coinfection with R. conori in 75 % cases. A- in the first phase, one or more primo affects (echara) develop, which exulcerate. In the second phase with a pronounced general infectious syndrome, B-occurs generalized papulovesiculous exanthema, which lasts a long time***

Thutsugamushi typhus (scrube typhus) bears a common taxonomic name, Orientia (O.) or Rickettsia tsutsugamushi, despite its great heterogeneity. They are very different from the Rickettsia typhus group. In our country, these pathogens have not been diagnosed, and they are mentioned due to the real possibilities of import and creation of new endemic foci. They are widespread in Asia, Australia, and the Pacific islands.

The vectors are larval forms of fleas and mites. Fleas and rodents are basic reservoirs. Transovarian transmission of rickettsiae in the flea population has been demonstrated. Human infections occur accidentally, by including humans in the flea food chain. Incubation is 1-3 weeks. Patients with typhus scrub often have only fever, headache and enlarged lymph nodes. Generalized adenopathy is unique among rickettsial diseases. Myalgia, gastrointestinal upset or cough are often present. At less than 50% cases local exanthema (Eshar) occurs, at the feeding site of thrombiculated mites (cigger) and classical exanthema, which spreads centrifugally. By penetrating the skin, the causative agent damages the microcirculation of the skin (rash), lungs (pneumonitis), brain (encephalitis) and other organs. Phagocytosed O. tsutsugamushi in an infected host escapes from the phagosome into the cytosol, divides by binary fission, and is released across the cell membrane. The pathogenic mechanism of action of O. tsutsugamushi is not fully known. Mortality averages 7% in untreated patients. The therapy of choice, like in other rickettsioses, are tetracyclines and chloramphenicol. There is no specific prevention.

Other rickettsial diseases and closely related diseases with rickettsial diseases

In the past 30 years, many new rickettsial agents have been detected in ticks using molecular techniques from different parts of the world. Some of these rickettsiae: R. slovaca, R.

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aeschlimannii and R. rhipicephali, were found in the former Yugoslavia (in Croatia). R. slovaca, R. helvetica, R. aeschlimannii, R. raoultii have been found in different parts of the world.

On the other hand, Q fever, ehrlichiosis/anaplasmosis, bartonelosis, which have similarities but also differences in relation to vasculotropic rickettsiosis, no longer belong to the order Rickettsiales, but are classified as closely related diseases with rickettsiosis.

Debonel / Tibola / Senlat

The dominant symptoms of new rickettsial syndromes are characterized by: inoculation rash, after tick bites, painful regional adenopathy, fever. Exanthema is rare. Alopecia remains at the site of scar healing. Debonel/Tibola/Senlat are also found under the name Dermacentor borne necrosis erythema, lymphadenopathy-tick-borne lymphadenopathy, and SENLAT (scalp echar and neck lymphadenopathy after tick bite). A tick bite is always found on the hairy part of the head. The causes are different types of rickettsiae. For now, R.slovaca and R.raoulti have been identified, and the tick carrier Dermacentormarginatus.

In Hungary, 27 cases of this infection have been described, which seems to be geographically limited. In almost all patients, tick bites occurred in the region along the banks of the Danube. Diagnosed causative agents are R. Slovak and R. raoulti

It is also described in Spain. In the period from 1990-2004. at La Rioja Hospital in Spain, 54 patients with this syndrome were observed. In all cases, tick bites were on the hairy part of the head (90%). The incubation period was 4 -7 days. Only a small number of patients had mild non-specific blood disorders, moderately elevated sedimentation and CRP, proteins and liver enzymes. Examination of patients and ticks, sequence obtained by PCR, revealed in 98% the identity of Rickettsia spp. (R.slovaca and R.raoulti). (Figures 7 and 8)

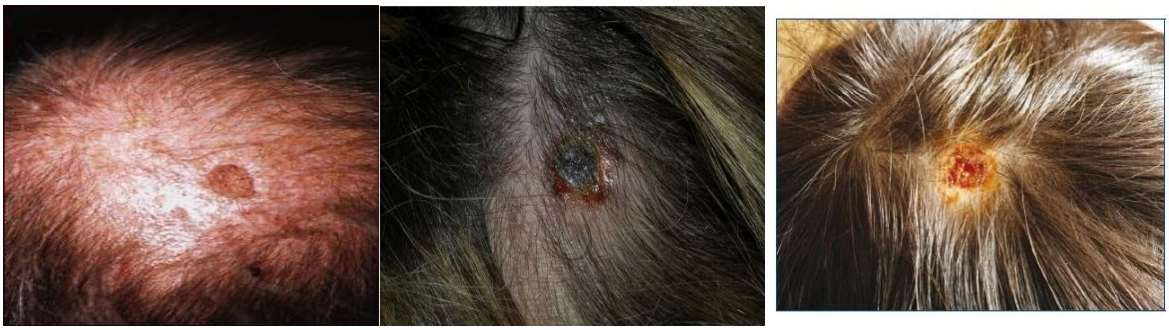

Figure 7. ***A-scalp echar and neck lymphadenopaty after tick bite***

In 90% of cases the tick sting is on the scalp. Up to this momment, the diagnosed etiological agents are R. slovaca and R. raoulti; A- scalp echar and neck lymphadenopathy after tick bite, B-Dermacentor borne necrosis erythema, lymphadenopathy - tick borne lymphadenopathy

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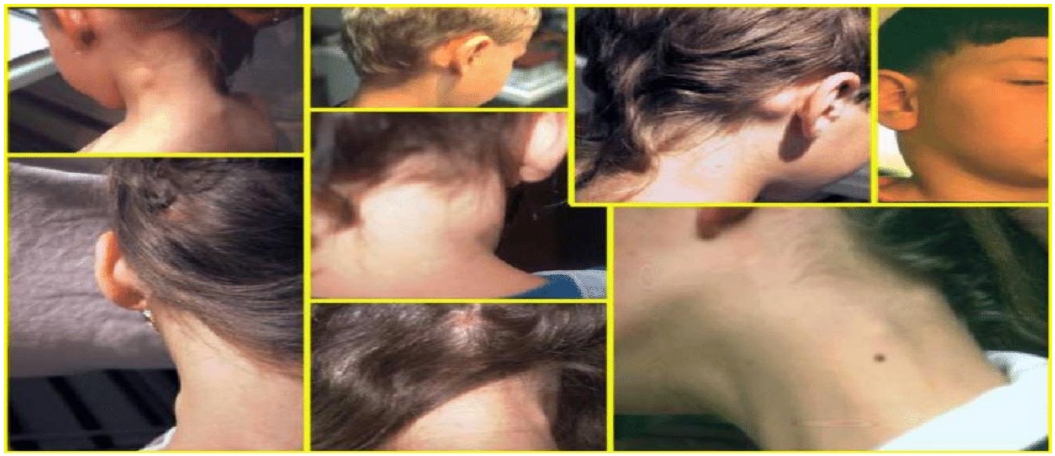

Figure 8. ***B- Clinical characteristics of the new rickettsiosis Tibola / Debonel / Senlat, transmitted by the tick Dermacentormarginatus.***

Coxielosis

Coxielosis (Q fever) is widespread worldwide. The causative agent, Coxiellaburnetti (Cb) is classified as a dangerous cause of infectious diseases, regardless of whether it is a subclinical (in 60% of cases), acute or chronic infection. Clinical manifestations vary, from benign, self-limiting syndromes, to severe interstitial pneumonias, which are typical in the acute phase, hepatitis with or without jaundice. Endocarditis is one of the extremely severe and dangerous manifestations. It usually affects people in the chronic phase of the infection. The spectrum of CNS syndrome also belongs to the extremely dangerous manifestations, which can endanger the life of patients. Other organs may also be affected. Immunodeficient people are endangered due to the development of severe, chronic forms of the disease.

In Montenegro during 1995/1996 the first 10 cases of Q fever in humans have been serially confirmed. In the same period, the prevalence of coxielosis in animals was estimated at 0.29% in the total livestock (sheep) fund, noting that the problem is certainly much more important**.** In the period from 1996 to 2017, 2450 patients` serums were tested for VBD agents, primarily co-infectious forms of Lyme borreliosis (LB). Out of the 158 subjects diagnosed with Coxyelosis, in 10% of cases it was registered in the co - infectious form of Lyme borreliosis (LB). Veterinary tests on serum of domestic animals in 2016/17, revealed coxielosis in 251 sera in cattle, 54 sera insheep and only in 5 diseased goats. Patients with Q fever were examined and treated at the University Clinic for Infectious Diseases in Podgorica.

Ehrlichioses/Anaplasmoses

The first etiological confirmations of ehrlichiosis during our examinations were obtained in the period from 2008-2017. By IIF and PCR methods, ehrlichiaacanis was diagnosed in 64 subjects, presented as human granulocytic ehrlichiosis (abb. HGE).

Based on the homology of the 16S rRNA sequences, ehrlichiae are genetically related to Rickettsia. Dogs, cats, rodents are the primary reservoirs of ehrlichia. These gram-negative

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bacteria, like morulae, are located inside the cytoplasmic vacuoles. Ehrlichiae are primarily recognized as diseases of animals and then humans, with the possibility of establishing a permanent infection. Later, 3 basic forms of the disease were identified: human granulocytic ehrlichiosis (HGE), human monocyte ehrlichiosis (HME) and Senets fever.

The first case of HME was reported in the United States in 1987. Infections ranged from severe to fatal, mimicking Rocky Mountain Spotted Fever, to oligosymptomatic or asymptomatic forms. A history of tick bites, seasonal and geographical distribution, in the absence of others, are important criteria for setting suspicion and conducting diagnostics. The disease is often accompanied by leukopenia, thrombocytopenia and liver damage. Lesions include perivasculitis in the central nervous system, kidneys, heart. Granulomas form in the lungs, bone marrow, liver. The clinical diagnosis is difficult. Laboratory diagnostics by the IIF method and PCR are the methods of choice for diagnosis. Morulae are difficult to detect in peripheral blood leukocytes. In 1994, granulocytic ehrlichiosis (HGE) was first reported, when ehrlichiae within the morula in neutrophils in peripheral blood smears, were indentified. It is very closely related to E.phagocytophile, the cause of European infection of sheep, cattle, goats and deer transmitted by ticks and E. equi (horse diseases). Different types of ticks transmit human HE. Laboratory diagnosis is made by IIF and PCR methods. Treatment with doxycycline is generally effective, but it has been proven that ehrlichie can also establich permanent infections in humans. Ehrlichia sennetsu in Japan causes a human disease (Senetsu fever), with is similar to infectious mononucleosis. Ehrlichiosis is clinically characterized by a sudden onset, with a pronounced general infectious syndrome, acute gastroenteritis, respiratory symptoms, CNS manifestations (meningitis). Measles rarely occurs. Leucopenia, thrombocytopenia, anemia, slightly to moderately increased activity of serum transaminases and alkaline phosphatase are registered in the blood picture. It lasts three weeks. Due to non-specific, and sometimes more severe clinical manifestations, differential-diagnostic should be distinguished from: septic shock, toxic shock syndrome, thrombocytopenic purpura, viral hepatitis, cholangitis. Over 60% of patients require hospital treatment. Fulminant forms of the disease are registered in immunodeficient, splenectomised patients. In these patients, particulary severe coinfections are risky.

Pathoanatomical changes are characterized by vasculitis, perivascular lymphocyte infiltrates, tissue infiltration with mononuclear cells, including nonspecific granulomas and hypercellular morulae. The disease resembles freckles, with the proviso that exanthema does not occur in all patients (it is registered in about 80% of patients). Leukopenia is a consequence of leukocyte destruction. Mortality is up to 5%.

Anaplasmosis was first described in 1994. The causative agents are Anaplasmae. Ixodes ticks are vectors, and reservoirs are smaller mammals, including mice in rural and suburban areas. The disease is similar to HME, but with a higher mortality rate (up to 10%). Laboratory diagnosis, prevention therapy, and control are similar to HME. Several types of live and dead vaccines have been used to establish control of anaplasmosis in cattle. These vaccines have been shown to be effective in preventing clinical manifestations of anaplasmosis in cattle, but do not block infection. Consequently, persistently infected cattle represent reservoirs of infection, through

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infectious blood both for mechanical transmission (contact), and especially for tick infection. Out of the 64 cases diagnosed with ehrlichiosis (E. canis), 73% had tick bites and 27% had no tick bites.

Bartonellosis

The disease has been known for a long time, based on the clinical picture of Cat Scrach Disease (CSD) and the presumed connection with animal (natural) reservoirs of the causative agent. Evolutionary progress and changes in the properties of the infectious agent have classified it as a dangerous disease, which is reflected in the increase of the number of clinically manifested infections, increase in the number of severe disseminated forms of infections, participation in coinfections, numerous problems of therapy, prevention, prognosis. In the last two decades, the frequency of diagnosed cases in the world has been increasing. There is also an evident correlation with diseases and conditions of immunodeficiency (AIDS and others). Natural reservoirs for B. bacilliformis and B. hensley are known (cats, rodents). For some bartonelles they are not known. Phlebotomies, sandflies and other biplanes are vectors in human infections.

During our tests, Bartonella henselae was first confirmed in 2007 in 11 cases, by the IIF method, in patients with the clinical picture of cat scratch disease (CSD). By the end of 2017, Bartonella henselae was confirmed in total of 42 cases. In addition to the most commonly diagnosed cat scratch disease (CSD), the first cases of hepatic pelidosis (HP) and bacillary angiomatosis (BA) were diagnosed in our immunodeficient patients. Based on genetic tests, the isolated bartonella genus was classified into speciese. Bartonella is gram-negative bacillus, identified as VBD causes of human infections 1990 (B.henselae), 1992 (B quintana). Later, from a wide range of bartonella agents, many new species were confirmed. They cause various infections in humans, and recent studies have confirmed the participation of various bartonella spp. and subspecies in co-infections with a spectrum of zoonotic agents of the VBD complex. It has also been proven that in natural hosts and vectors, bartonella can exchange its genetic material with numerous and different infectious agents. Bartonellae are widespread in the world, especially in areas with warm and humid climates. In the last two decades, cases with a manifest and severe clinical picture have become more frequent. The competence of the host immune system determines the severity and spectrum of clinical manifestations of the disease. During our studies, the evident correlation with diseases and conditions of immunodeficiency (AIDS) was confirmed. Cats and rodents are a natural reservoir for B. bacilliformis and B. hensley.The cat flea Clenocephalidesfellis is the primary vector for the transmission of the infectious agent in the human population. About 40-60% of cats have asymptomatic B. hensley infection, associated with bacteremia.

For most bartonellae, polytropism is characteristic. They have an affinity and attack different types of human cells. The mass of bartonella fills the cytoplasm of cells along the blood vessels, and the thickening of the endothelium of the blood vessels results in vascular occlusions and thrombosis. The clinical picture of the first phase of the disease is dominated by the symptoms of the general infectious syndrome.

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In the second phase, these symptoms during bacteremia can develop: febrile recurrent endocarditis, septicemia, neurological, psychiatric, ophthalmic disorders and severe hemolytic anemia, after incubation for 10-14 days (Figure 9, 10,11, 12, 13, 14)

Figure 9. ***In CSD at the site of penetration of infectious agents, primo affect develops (Original photo documentation by Prof. Dr. B. Andric)***

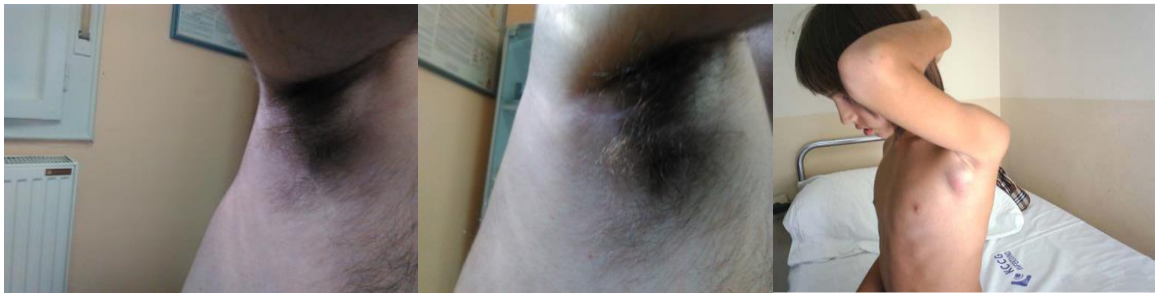

Figure 10. ***Adenopathy, becomes noticeable in 3 - 10 days, in 50% of patients and is usually accompanied by fever, chills, malaise, headache, conjunctivitis, pharyngitis, anorexia. Often painful regional adenopathy, persists for weeks or months. (Original photo documentation of Prof. Dr. B. Andric)***

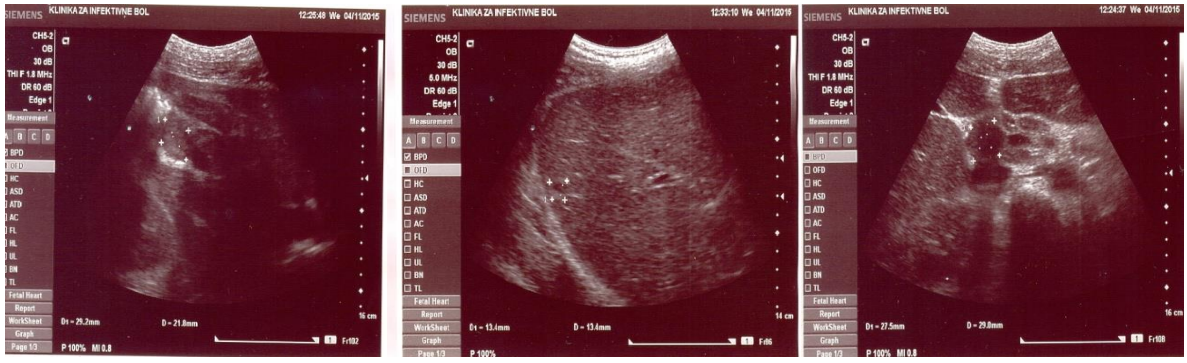

Figure 11. ***Hepatic pelidosis was diagnosed in 4 cases. Ultrasound diagnostics registered enlargement of the liver and spleen and conglomerates of enlarged lymph glands in the hilar and parapancreatic region with pronounced symptoms of general infectious syndrome (Original photo-documentantionbyProfdr.B.Andric)***

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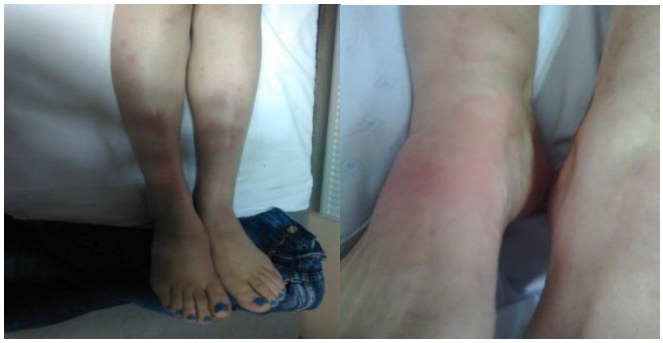

Figure 12. ***In 2 cases with CSD, in the acute phase, in addition to the pronounced symptoms of the general infectious syndrome, changes in erythema nodosum were manifested. Original photo documentation Prof. Dr. B. Andric)***

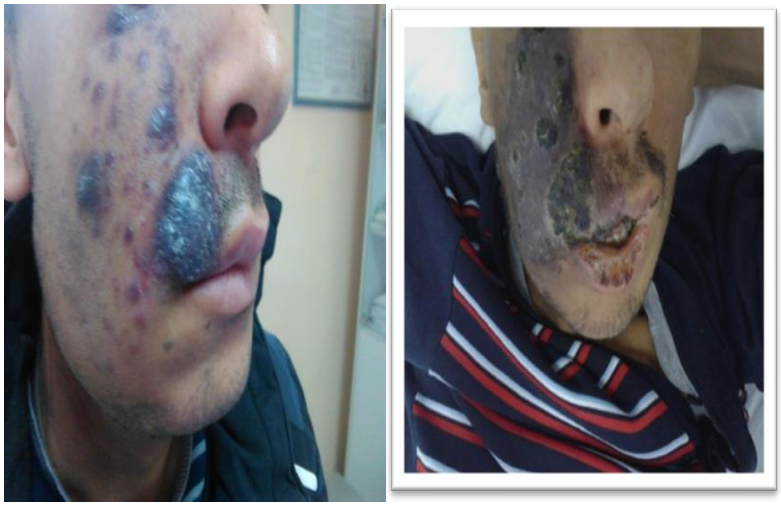

Figure 13. ***In patients with severe immunodeficiency, severe forms of bartonellosis occur. At our Clinic, two cases of bartonelosis with HIV / AIDS coinfection were diagnosed***.

In these patients, bartonelosis/AIDS coinfection manifested itself with skin lesions reminiscent of angioproliferative lesions of Kaposi's sarcoma. The pathohistological finding removed the dilemma by showing that it is a case of vascular proliferation of capillaries with typical endothelial cells with edema and neutrophilic basophilic infiltrates (Original photo documentation by Prof. Dr. B. Andric)

COINFECTIONS

Our studies in Montenegro have shown a frequent participation of rickettsial agents in epidemiologically conditioned co-infections with Borrelia burgdorferi. (Chart 1).

In the period from 2015/16, 2450 patient sera were examined. Co-infective forms of LB were diagnosed in 956 patients (83.93%). In the group of epidemiologically conditioned co-infections with B. burgdorferi, a total of 582 cases, the most frequently registered rickettsial agents were in

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453 cases (R. conorii 69%, E.canis in 47%, C.burnetti 10%, Bartonella 19 cases + Bartonella henselae in 19 cases.)

Simultaneous infections of ticks, humans and mice with several types of VBD complex agents have been proven by molecular biology methods. In our research, in co-infections among agents of the VBD complex, in 20% of cases, microbiological studies revealed the participation of Brucella spp. The results came as a surprise given the rigorous veterinary measures in controlling brucellosis.

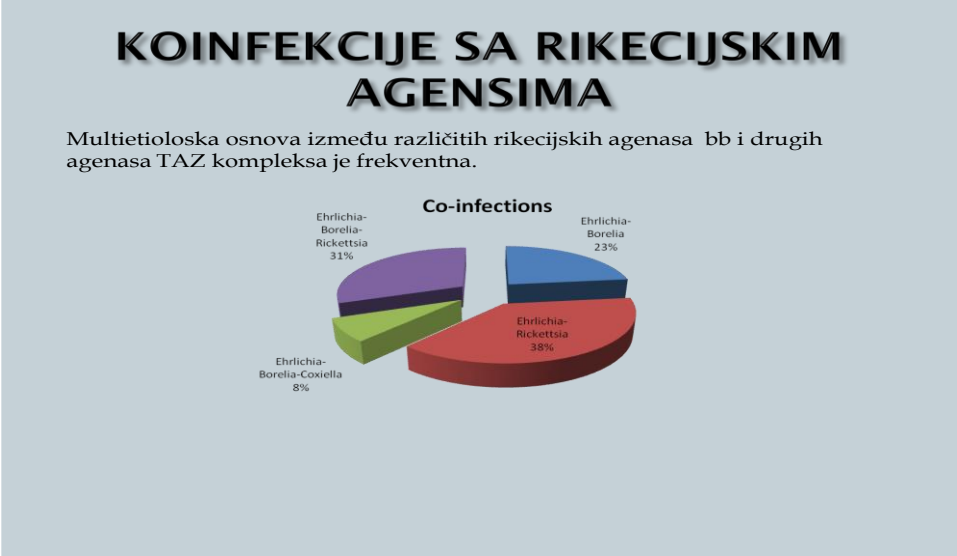

Chart 1. ***Co-infections with rickettsial agents***

Epidemiological studies have clarified our dilemmas, given that there were two sources of imported brucellosis from the surrounding countries (Albania and Bosnia and Herzegovina). Coinfections between C. burneti and Brucellae have been diagnosed worldwide. Three sets of blood cultures were microbiologically processed. Gram-stained, small Gram-negative cocobacilli were detected. Using the Vitek ON identification card, the isolated microorganism was identified as Brucella melitensis. By sequencing the 16 S sRNA gene isolate, Brucella spp was confirmed, the Rose Bengal test was positive, while Wright agglutination showed a significant increase in antibody titer of 80 - 640 in paired sera. With the IIF test, the titer indicated acute IgM antibodies to C. burnetti, phase II, which is evidence of co-infection of C. burnettiand Brucellamelitensis.

It is known that the types of participants in epidemiological co-infections are determined primarily by the geographical distribution of different VBD agents in endemic foci and depend on numerous factors: changes in vector properties, infectious agents, host susceptibility.

DISCUSSION

During the last 30 years, the expansion of some old and the appearance of new rickettsiae outside the known endemic foci with the appearance of atopic rickettsiosis has been registered (in France, R. slovaca and R. mongolotymone are now in prevalence). Since the epidemic

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occurrence of known rickettsial diseases (2-9), the appearance and expansion of the epidemic typhus in the world since 1970, and its appearance in areas where it did not *exist* before, with a high mortality rate are of special importance. The drastic increase in Spotted fever rickettsial diseases (SFRD) in the late 1970s, ehrlichiosis since 1990, the appearance of numerous new and atypical rickettsial diseases in human pathology, testify to the close connection of these diseases with drastic changes in the ecological environment. An increasing incidence of rickettsial fever and other rickettsial diseases is registered, especially in endemic areas of southern Europe, Africa and Asia (10-13).

Novelties in the study of rickettsial agents represent the knowledge that in common endemic areas, in natural hosts and vectors, they can exchange their DNA, or certain other functions, proteins, plasmids among rickettsial agents, but also with the other members of the VBD complex (borrelia, babesia, bartonella, etc. . Furthermore, they can even carry on the exchange with a wider range of agents that do not belong to this complex (brucellae). Natural reservoirs and vectors are "natural laboratories" in which these processes begin and take place, but they also continue in the infected organism.

The consequent increase in adaptability has enabled an increase in the number and species of new rickettsial agents, an increase in the spectrum of natural hosts and vectors, an increase in the ability of old and new agents to move from enzotic to zoonotic transmission cycles, without the need for longer evolution in enzotic cycles. Multietiological transmission of infectious agents through the same vector, multi etiological basis of human infections, affect the course, severity of the disease and the occurrence of complications. These natural epidemiological changes continuously provide new opportunities in the evolutionary movements of rickettsial and other agents of the VBD complex, which are responsible for the creation of new species and subspecies.

It is known that the types of participants in epidemiological coinfections are determined primarily by the geographical distribution of different agents of the VBD complex in endemic foci and depends on numerous factors: Changes in vector properties, infectious agents, host susceptibility. It is not clear which factors change the affinity of different types of ticks towards the hosts on which they feed. Are such changes a consequence of climatic influences (precipitation, floods, droughts, global warming, etc.)? Recent research has confirmed the importance of climatic influences, especially on the abundance and distribution of Ix sangvineus, the vector of R. conorii-the cause of Mediterranean spotted fever. This tick is suitable for high temperatures, low rainfall and drought, which affects the occurrence of the disease throughout the year, while frequent rainfall, accompanied by low temperatures and humidity, significantly reduces the population of this type of tick and the occurrence of the disease.

Montenegro belongs to the group of Balkan and Mediterranean countries. Considering the geographical localization, climatic conditions, characteristics of flora and fauna, the occurrence of rickettsial diseases is expected, which is indicated by historical data and modern studies of rickettsial diseases in our environment. Montenegro and Serbia were the last countries in Europe

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to report cases of epidemic typhus after World War II. Since1946. Epidemic typhus is not registered in Montenegro, but in the light of recent findings, this disease cannot be considered to belong to the history of medicine, given the proven possibilities of interepidemic maintenance in enzotic cycles, its occurrence and expansion in the world (Burundi, Peru, Algeria), and disease outbreaks in countries where it has not been present.

The potential animal reservoirs of R. prowazecki have not been studied in Montenegro, although it is known to be an endemic area for this rickettsiosis, with the possibility of reappearing as a plague in modern society.

Significant qualitative and quantitative changes in epidemiological and immunological properties in the natural cycles of TAZ, have led to the expansion and globalization of rickettsiae as well as the emergence of numerous new species of rickettsial agents, especially in the group of spotted fevers. , the discovery of competitive relationships between infectious agents of the group of transmissible zoonoses (bb, rickettsiae, ehrlichia, babesia) in arthropods and infections (co-infections), represent the epidemiological basis, which with complex pathogenetic and immune pathogenetic mechanisms of infection / co-infection diseases.

CONCLUSION

Natural conditions classify our country in extremely suitable areas for the existence of diseases from the group of VBD, among which Rickettsiae occupy a significant place. Our studies have shown that in common endemic areas, the most frequently associated infections are **bb** and various tick-borne rickettsial agents. Based on studies conducted in Europe, ehrlichiae are ranked among the most common transmissible agents transmitted by ticks with **b. burgdorferi**, but also other agents of the TAZ complex are also frequent. The geographical distribution of VBD complex agents determines the participants in co-transmission through the same vector as the co-infection participation of VBD agents in multi etiological (co-infection) forms of TAZ, which on the other hand complicates the diagnosis, therapy and prognosis of the disease. Examinations by electron microscopy have shown that the rickettsial agents are surrounded by a poorly characterized structure, and are thought to represent a mucosa or capsule rich in polysaccharides. The cell wall contains lipopolysaccharides (LPS), the main component of pathogenicity and the difference between rickettsiae of typhus group and rickettsial spotted fevers. Complex antigenic structure, high adaptability and resistance in the environment, allow rickettsial and related agents to continuously change and create new species, which very easily, and without the need for long-term evolution in enzotic cycles, turn into zoonotic cycles, causing severe human diseases. These subtle and long-lasting changes cannot be predicted or controlled, which classifies rickettsial agents as threatening dangers for the future.

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