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Emerging tick-borne spotted fever group rickettsioses in the Balkans

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Abstract

The impact of tick-borne pathogens (TBPs) on human health has increased in the last decades, since the incidence of emerging and re-emerging infectious and zoonotic tick-borne diseases has increased worldwide. Tick-borne rickettsiae of the Spotted Fever group (SFGR) are considered as emerging pathogens that can infect humans and cause a variety of non-specific clinical symptoms. Here, we report nine cases of atypical tick-borne diseases (9/460; 1.95%) that occurred over a period of four months (from 15 April 2021 to 16 August 2021) in Serbia, from which five cases were classified as confirmed SFGR infection, two cases as probable SFGR infection and two cases as suspected SFGR infection. Within cases of confirmed SFGR infection, *R. helvetica* was detected as the causative agent in two cases. The most common clinical finding was non-expanding persistent circular redness, followed by eschar and enlargement of regional lymph nodes, and pain at lesion site. *Rickettsia* outer membrane protein B (*ompB*) and citrate synthase (*gltA*) gene fragments were amplified from clinical samples and ticks attached to patients and IgG reacting with *Rickettsia conorii* antigen were detected in sera samples of patients, which are highly suggestive of exposure to SFGR. Surveillance and monitoring of rickettsial diseases in Serbia should continue and extended to new areas due to the increasing trend of clinical infections caused by SFGR in the country.

Keywords: Ticks, Spotted Fever Group Rickettsia, emerging diseases

1. Introduction

The genus *Rickettsia* (Rickettsiales; Rickettsiaceae) comprises gram-negative, obligate, intracellular, and short rod-shaped bacteria, which are transmitted by arthropod vectors such

as lice, fleas, ticks and mites (Fournier and Raoult, 2007). The system of classification of rickettsiae has re-organized *Rickettsia* spp. into four major groups that include 1) spotted fever group rickettsiae (SFGR), 2) typhus group rickettsiae (TGR), 3) ancestral group rickettsiae (AGR) and (4) transitional group rickettsiae (TRG) (Merhej et al., 2014). The heterogeneous SFGR contains more than 20 rickettsiae species, mainly transmitted to humans by ticks, except for *Rickettsia felis* and *Rickettsia akari* which are transmitted by fleas and mites, respectively (Parola et al., 2013). Interestingly, both *R. felis* and *R. akari* have been proposed to be grouped into TRG from a host perspective, since ticks are not considered as a vector for neither of these *Rickettsia* species (Diop et al., 2020; Gillespie et al., 2007).

In Europe, tick-borne rickettsioses are the main source of rickettsial infections in humans, which may cause mild to severe infection and represent a major concern to public health (Portillo et al., 2015). The most common clinical features of rickettsioses in humans are fever, headache, rash, and inoculation eschars (Fang et al., 2017). In contrast to the high prevalence of SFGR infection in ticks across the Europe, human cases of SFGR infections are not commonly reported. Possibly, the main reason for this phenomenon is low virulence, leading to subclinical or self-limited mild manifested infections that are often unrecognised by medical professionals, particularly if the patient is not aware about previous tick encounter (Azagi et al., 2020; Jensenius et al., 2006). The other possible reasons for underreporting of SFGR infections in humans outside endemic areas are absence of specialised laboratories, absence of diagnostic guidelines and/or unawareness of medical professionals about the risk of SFGR infection after tick bites (Jensenius et al., 2006; Kostopoulou et al., 2016). This may explain why the majority of reported human cases, other than Mediterranean Spotted Fever (MSF), are reported in territories where specialised facilities for SFGR research and/or diagnostic are present (Dubourg et al., 2014; Kostopoulou et al., 2016; Nilsson, 2009).

Consequently, SFGR infections in Europe outside areas endemic for MSF may be highly neglected (Jakimovski et al., 2022).

Surveillance and reporting procedures for SFGR infections are not standardized across Europe and in countries where notification exist, it is not always mandatory. This may affect the estimation of the disease prevalence in certain European regions, such as the Balkans, where the first case of *Rickettsia sibirica mongolitimonae* infection has been recently described in North Macedonia (Jakimovski et al., 2022), and SFGR infection with atypical presentation have been described in Serbia (Banovic et al., 2021b). In the present study, we report a series of confirmed, probable and suspected SFGR cases that occurred between 15 April 2021 and 16 August 2021 in Serbia, and nearby territories. Genetic analysis of the bacterial genes *gltA* and *ompB* showed genetic diversity associated with identified SFGR pathogens.

2. Materials and methods

2.1. Ethics statement

This investigation was approved by the ethical committee of Medicine Faculty Novi Sad, University of Novi Sad (Ethical approval No. 01-39/24/1) and conducted according to Declaration of Helsinki, The Patient Rights Law of the Republic of Serbia, and EU Directive 2010/63/EU on animal experimentation.

2.2. Clinical diagnosis of tick-borne rickettsial infections

The diagnosis of tick-borne rickettsial infections was conducted in accordance with the Guidelines of the European Society of Clinical Microbiology and Infectious Diseases guidelines (ESCMID) (Brouqui et al., 2004). More specifically, patients who developed rash,

eschar or non-expanding persistent redness (at the site of the tick bite) with or without regional lymphadenopathy / lymphangitis 72h after tick removal were considered as potential SFGR disease cases and underwent additional examination. In addition to ESCMID guidelines, development of non-expanding redness accompanied with itching sensation was also considered to be indication for SFGR disease, since those were the signs and symptoms developed after formation of eschar in suspected SFGR infection case described previously in Serbia (Banovic et al., 2021b). The molecular and serological analyses described under the sections below, “DNA microfluidic real-time PCR assay” and “Indirect immunofluorescence assays”, were conducted solely for the purpose of retrospective assessment of the clinical diagnosis.

2.3. Antibiotic treatment in patients with clinical signs of rickettsial infections

The patients who presented any of the abovementioned signs (i.e., eschar, and non-expanding persistent redness) or symptoms (i.e., itching sensation at the skin affected by lesion) related with SFGR infection were prescribed an antibiotic therapy including 100 mg doxycycline (Dovicin, Galenika AD, Serbia) twice per day for 10 days. The molecular and serological analyses described below were not considered in any way, shape or form to inform the decision of therapeutic intervention.

2.4. Collection of clinical samples

Immediately after SFGR etiology was suspected, capillary blood samples were collected from the center of lesion (the tick bite site) of all patients. Firstly, the tick bite site was visualized, then the skin was cleansed with antiseptic, pricked with a lancet and the capillary blood collected in a sterile cotton swab, that was later used for DNA extraction. Capillary blood samples were used for direct detection of SFGR genetic material (see below the sections “*DNA microfluidic real-time PCR assay*”). If eschar was present at the time of clinical

examination, a sample from the eschar (i.e., eschar crust) was also collected and stored in sterile vial. Eschars crusts were used for direct detection of SFGR genetic material (see below the section “*DNA microfluidic real-time PCR assay*”). Whole venous blood samples for serological examination were collected 4 weeks after tick removal or, in cases that patients were not aware of tick bites and/or tick encounter, after appearance of characteristic lesions such as eschars. Whole blood sample was used for serum extraction and serological examination (see below the section “*Indirect immunofluorescence assay (IFA)*”).

2.5. Tick collection from patients and taxonomic identification of ticks

Upon arrival in the medical consultation, attached ticks were collected from patients using tweezers. In some cases, the ticks were removed by the patients before arriving at the medical consultation and the ticks were handled to the physician. All collected ticks were classified at the genera and/or species levels based on morphological features according to standard taxonomic keys described by Estrada-Peña et al. (2004). Ticks were also classified according to sex, and life stage (i.e., larvae, nymphs and adult male or female). Attachment time was inferred from approximate feeding time, assessed according to coxal index (for tick attachment suspected to last less than 24h) and scutal index (for tick attachment suspected to last 24h or more), as described by Gray et al. (2005) and anamnestic data related to patient activities. In addition, the molecular identification of *Ixodes ricinus* and *Dermacentor reticulatus* was based on the detection of species-specific fragments of internal transcribed spacer 2 (*its2*) region of nuclear ribosomal DNA gene using the microfluidic real-time PCR as previously described Michelet et al. (2014). Attachment time of tick species other than *I. ricinus* was assessed via anamnestic data related to patient activities in nature and/or green surface area. Locations where patients were exposed to tick bites was inferred by combining data related to tick feeding time and anamnestic data provided by patients about activities performed during the given period.

2.6. Identification of components of potential chain of SFGR infection in household at risk

In cases where patient provided information about possible tick acquisition from a household animal, a field sampling session was organized to identify components of the chain of infection between humans, animals and ticks within households following the One Health tenets. Household animals were inspected for presence of tick or flea infestation, and venipuncture was performed in order to detect possible presence of tick-borne pathogens (TBPs) in whole blood. During the visit, household family members were asked to participate in study. Upon acceptance, whole blood samples were collected and analyzed for presence of *Rickettsia* spp. DNA and anti-SFGR antibodies (see below).

2.7. Indirect immunofluorescence assay (IFA)

The fraction of the whole blood samples placed in vials without anticoagulants was allowed to clot at room temperature. After 2000 ×g centrifugation for 10 min, serum samples were collected and inactivated at 56 °C. The reactivity of sera IgG to SFGR antigens was tested using a commercial IFA that uses *Rickettsia conorii* as the source of SFGR antigens (Vircell, Spain, Ref. 'PRICOG'). Since paired sera samples were not available, a single-point serological test was used here to assess the presence of anti-SFGR IgG in sera samples diluted 1:32, 1:64, 1:128, and 1:256. Visible fluorescence reaction with sera diluted $\geq 1:64$ was considered as positive (Biggs et al., 2016; McQuiston et al., 2014).

2.8. Blood parameters analysis

In order to assess infection severity and/or systemic damage, before initiation of antibiotic treatment, or within 24h after treatment was initiated, patients suspected of SFGR infection were asked to assist Certified Medical Laboratories (i.e., approved by Serbian Ministry of Health) of their choice to test blood parameters including the levels of aspartate

aminotransferase (AST), alanine transaminase (ALT), gamma-glutamyl Transferase (GGT), C-reactive protein (CRP), lactate dehydrogenase (LDH) and creatine kinase (CK), as well as complete blood count (i.e., number of red blood cells, white blood cells, and platelets, as well as levels of hemoglobin and hematocrit).

2.9. DNA extraction

Total DNA was extracted from clinical samples (i.e., capillary blood and eschar crusts) and tick specimens using the Nucleospin Tissue kit (Macherey Nagel, Düren, Germany), according to the manufacturer's instructions, and as described previously (Banovic et al., 2021b). Extracted DNA was stored at -20°C until needed.

2.10. DNA pre-amplification

To improve the detection of pathogen DNAs, extracted DNAs were pre-amplified with the PreAmp Master Mix (Fluidigm, San Francisco, CA, USA) used according to the manufacturer's instructions. Primers targeting all pathogens (see next section) were pooled by combining an equal volume of each primer for a final concentration of 200 nM. The reaction was performed in a final volume of 5 μL containing 1 μL Perfecta Preamp 5X, 1.25 μL pooled primer mix, 1.5 μL distilled water and 1.25 μL DNA. The thermocycling program consisted of one cycle at 95°C for 2 min, 14 cycles at 95°C for 15 s and 4 min at 60°C . At the end of the cycling program, the reactions were diluted 1:10 in Milli-Q ultrapure water. All the pre-amplified DNA samples were stored at -20°C until needed.

2.11. DNA microfluidic real-time PCR assay

The BioMarkTM real-time PCR system (Fluidigm, San Francisco, CA, USA) was used to detect major TBPs [27 bacterial species (*Borrelia burgdorferi* sensu stricto (s.s.), *Borrelia garinii*, *Borrelia afzelii*, *Borrelia valaisiana*, *Borrelia lusitaniae*, *Borrelia spielmanii*, *Borrelia bissettii*, *Borrelia miyamotoi*, *Anaplasma marginale*, *Anaplasma platys*, *Anaplasma*

phagocytophilum, *Anaplasma ovis*, *Anaplasma centrale*, *Anaplasma bovis*, *Ehrlichia canis*, *Neoehrlichia mikurensis*, *R. conorii*, *Rickettsia slovaca*, *Rickettsia massiliae*, *Rickettsia helvetica*, *Rickettsia aeschlimannii*, *Rickettsia felis*, *Bartonella henselae*, *Francisella tularensis*, *Francisella*-like endosymbionts, *Coxiella*-like endosymbionts, and *Coxiella burnetii*), 7 parasite species (*Babesia microti*, *Babesia canis*, *Babesia ovis*, *Babesia bovis*, *Babesia caballi*, *Babesia venatorum*, and *Babesia divergens*), 5 bacterial genera (*Borrelia*, *Anaplasma*, *Ehrlichia*, *Rickettsia*, and *Mycoplasma*), 3 parasite taxa (Apicomplexa, *Theileria*, and Hepatozoon)] in clinical samples and ticks collected from patients, by the high-throughput microfluidic real-time PCR amplification using 48.48 Dynamic Array™ IFC chips (Fluidigm, San Francisco, CA, USA). These chips dispense 48 PCR mixes and 48 samples into individual wells, after which on-chip microfluidics assemble real-time PCR reactions in individual chambers before thermal cycling, resulting in 2,304 individual reactions. Briefly, amplifications were performed using 6-carboxyfluorescein (FAM)- and black hole quencher (BHQ1)-labeled TaqMan probes with TaqMan Gene expression master mix following manufacturer's instructions (Applied Biosystems, Courtaboeuf, France). PCR cycling comprised 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 2-step amplification of 15 s at 95°C, and 1 min at 60°C. One negative water control was included per chip. To determine if factors present in the sample could inhibit the PCR, *Escherichia coli* strain EDL933 DNA was added to each sample as an internal inhibition control, and primers and probe specific for the *E. coli eae* gene were used. For more details regarding the development of this new high-throughput tool based on microfluidic real-time PCR amplification (test of sensitivity, specificity, targeted genes, primers/probes sets and controls used), please see Michelet et al. (2014) and Grech-Angelini et al. (2020).

2.12. Validation of microfluidic real-time PCR assay

Rickettsia-positive samples were randomly selected to undergo additional conventional and nested PCR assays using primers different than those of the BioMark™ real-time PCR system (Choi et al., 2005; Masatani et al., 2017; Rar et al., 2005; Regnery et al., 1991). In particular, targeted fragments of *Rickettsia* outer membrane protein B (*ompB*) and citrate synthase (*gltA*) were amplified using primers and PCR conditions as previously described (Banovic et al., 2021b). Amplicons were sequenced by Eurofins MWG Operon (Ebersberg, Germany) and assembled using the BioEdit software (Ibis Biosciences, Carlsbad). The final nucleotide sequences were analyzed to identify the sequenced microorganisms using the GenBank database through the National Center for Biotechnology Information (NCBI; Bethesda, MD, USA) Basic Local Alignment Sequence Tool (BLAST) search engine (www.ncbi.nlm.nih.gov/blast). Nucleotide sequence data reported in the present study are available in the GenBank, EMBL and DDBJ databases under the accession numbers ON314263-ON314265, ON314267-ON314270.

2.13. Phylogenetic and genetic distance analyses

To assess the identity and genetic diversity of the *Rickettsia* species identified in this study, the obtained *gltA* and *ompB* sequences were analyzed using the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990). Once the obtained sequences were identified at the species level, the GenBank database (<https://www.ncbi.nlm.nih.gov/>) was searched for gene sequences within the same bacterial species and previously identified in different regions including Serbia, other European countries and when available other countries from Africa, Asia, America, and Australia. Sequences of the same gene and within the same bacterial species were grouped for phylogenetic tree reconstruction. Each sequence group was then aligned using MAFFT configured for the maximum accuracy. The best-fit models

of *gltA* and *ompB* sequences evolution in each bacterial species were selected based on the lowest values of Corrected Akaike Information Criterion (cAIC) and Bayesian Information Criterion (BIC), as implemented in Molecular Evolutionary Genetics Analysis (MEGA) X (Kumar et al., 2018). The Tamura 3-parameter (T92), and Jukes-Cantor (JC) models, which showed the lowest cAIC and BIC values, for *Rickettsia monacensis gltA* and *R. helvetica ompB* (JC), *Rickettsia raoultii gltA* (T92), *R. slovacica gltA* (JC), *R. helvetica gltA* (T92), and *R. raoultii ompB* (T92), were chosen for tree reconstruction. The phylogenetic trees were built using the maximum likelihood (ML) method using the option ‘complete deletion’ (i.e., Removal of unaligned positions) as implemented in MEGA X. Reliability of internal branches was assessed using the bootstrapping method (500 bootstrap replicates) implemented in MEGA X.

2.14. Genetic distance analysis

The genetic distances between the *Rickettsia gltA* or *ompB* sequences obtained in this study and other sequences in each phylogenetic tree were calculated as p-distance in MEGA X. Pairwise p-distance values resulting from three types of comparisons were compared to assess the genetic diversity of *Rickettsia* strains identified in this study and other *Rickettsia* strains previously identified in Serbia and other parts of world. The first set of comparisons involved the sequences identified in this study and others included in the trees (excluding sequences previously identified in Serbia). The second set of comparisons involved the sequences previously identified in Serbia and others included in the trees (excluding sequences identified in this study). Finally, all sequences in the tree were compared between them excluding sequences from Serbia obtained in this or previous studies. Sets of p-distance values were compared as described below.

2.15. Classification of SFGR infection cases

Following the retrospective assessment of diagnostic and xenodiagnostic findings combined with clinical manifestations observed in patients, SFGR infection cases were classified as: (i) confirmed SFGR infection – SFGR DNA was detected in eschar crust or in capillary blood sample collected from the lesion; (ii) probable SFGR infection – SFGR DNA is not detected in eschar crust or in capillary blood sample, but patient has compatible clinical manifestation with positive serology finding and positive xenodiagnostic finding (i.e., SFGR DNA was detected in tick removed from skin); and (iii) suspected SFGR infection - patient has developed compatible clinical manifestation with positive xenodiagnostic finding, but without positive serology or SFGR DNA detection in eschar crust or in capillary blood sample.

2.16. Statistical analysis

Differences in p-distance values between the analyzed DNA sequences were assessed in GraphPad software v.9 (GraphPad Software Inc., La Jolla, CA, USA). The Shapiro-Wilk normality test rejected ‘normal distribution’ of the obtained p-distance values. Therefore, the nonparametric Mann Whitney *U* test was used. Statistical significance was considered when $p < 0.05$.

3. Results

3.1. Clinical cases of SFGR

From total of 460 patients admitted to Pasteur Institute during year 2021 due to tick attachment and/or suspected tick-borne disease, only patients suspected of SFGR infection were included in the present study. All patients suspected of SFGR infection ($n = 9$) developed mild clinical presentation of disease (Table 1). The most common clinical finding

among the patients was non-expanding persistent circular redness that developed within 72h of tick removal, notably in patients 43/21, 106/21, 212/21, and 140/21 (Figure 1). Eschar and enlargement of regional lymph nodes were detected in 4 patients (i.e., patients 43/21, 45/21, 106/21 and 140/21; 4/9; 44.44%), while pain at lesion site occurred in 3 patients (i.e., patients 45/21, 140/21 and 427/21; 3/9; 33.33%). Systemic rash was not observed in any patient (0/9; 0%), while elevation of body temperature was registered in only one patient (401/21; 1/9; 11.11%), 5 days after doxycycline therapy was initiated. Detailed clinical case descriptions including relevant photographs of lesions are provided in Supplementary file 1. Blood parameters values were mostly within normal limits, but elevation of LDH and CK was detected in one and two patients, respectively (Table 1). All patients (9/9; 100%) described here achieved full recovery after doxycycline treatment. Notably, the 9 clinical cases of SFGR described here occurred over a period of four months (from 15 April 2021 to 16 August 2021), and they represent a significant increase in the number of SFGR cases (2021; 9/460; 1.95%), relative to the previous two years, 2020 (1/650; 0.15%) (Banovic et al., 2021b) and 2019 (0/480; 0%) (Banovic et al., 2021a). In the majority of described cases the location of exposure to tick bite was within rural areas on Fruška Gora Mountain (5/8; 62.5%), while Mali Idoš (North Bačka District), Beočin (South Bačka District) and Hašani (Bosnia and Herzegovina) were registered once each (1/8; 12.5%) (Table 1).

3.2. Retrospective assessment of SFGR infection using molecular and serological analyses

The presence of *Rickettsia* DNA was detected by microfluidic PCR in the samples collected from the eschar crust of patient 140/21 and the capillary blood of patients 43/21, 197/21, 212/21 and 401/21 (Table 1). In addition, the capillary blood samples of patients 43/21 and 197/21 were positive to *R. helvetica* by microfluidic PCR. The other eschar crusts and capillary blood samples were negative by microfluidic PCR to all tested pathogens. In

examined clinical samples no other pathogen than SFGR were found. In addition, no co-infection with two or more SFGR was detected by microfluidic PCR. Amplification and sequencing of a *Rickettsia ompB* gene fragment revealed the presence of *R. helvetica* DNA in the capillary blood of patient 43/21 (Table 1). The amplification of *R. helvetica ompB* or *gltA* in the sample of patient 197/21 was unsuccessful.

Exposure to bacteria of the SFGR group was further assessed by testing the presence of IgG reacting with *R. conorii* antigen in sera samples of 7 out of the 9 patients (Patients 45/21 and 140/21 were not available for sample collection) suspected of SFGR infection. Seroreactivity ($\geq 1:64$) to SFGR antigens was detected by IFA in 5 (5/7; 71.42%) patients, including 43/21, 197/21, 296/21, 401/21, and 427/21 (Table 1). Patients 43/21 and 197/21 were positive at the higher dilution used in this study (i.e., 1:256).

3.3. Presence of TBPs in ticks collected from SFGR patients

Of the 9 SFGR patients, 8 reported to Pasteur Institute Novi Sad with a previously removed tick or with the tick attached in their skin (8/9; 88.88%), and one patient (i.e., 140/21) did not notice any tick bite and therefore no tick was collected from this patient (Table 1). Based on morphological characterization, four tick species were identified infesting SFGR patients, being *I. ricinus* the most common (5/8; 62.50%, (3 adult females, and 2 nymphs), while *Dermacentor marginatus* (1 adult female), *D. reticulatus* (1 adult female) and *Haemaphysalis* sp. (1 nymph) were detected in one patient each. In addition to morphology, the identification of *I. ricinus* and *D. reticulatus* was based on the amplification of species-specific *its2* gene fragments. The shortest duration of tick attachment was estimated for an *I. ricinus* nymph (12h or less; patient 401/21), while average duration of tick attachment was 3.5 days (95% CI: 2.46 - 4.54). Tick species, developmental stage and feeding time of each SFGR case are presented in Table 1.

Microfluidic PCR analysis of DNA extracted from ticks removed from patients revealed the presence of *Rickettsia* DNA in all tick samples and *R. helvetica* in the ticks collected from patients 106/21, 197/21 and 212/21. Amplification and sequencing of *Rickettsia ompB* or *gltA* gene fragments confirmed the presence of SFGR DNA in the ticks collected from patients 43/21 and 45/21 (*R. raoultii ompB*), 197/21 (*R. helvetica gltA*), 296/21 and 401/21 (*R. monacensis gltA*), and 427/21 (*R. slovacica gltA*) (Table 1).

3.4. Sequence identity analysis of SFGR strains

The BLASTn analysis of *R. helvetica ompB* gene sequence (fragment size, 267 base pair (bp); accession number, ON314263) obtained from capillary blood sample of patient 43/21 revealed a high identity of 100% and 99.25% with sequences obtained from *R. helvetica* strains detected in an *I. ricinus* tick from Serbia (MW901478) and *Ixodes persulcatus* tick from Russia (KU310591). The BLASTn analysis of *R. raoultii ompB* gene sequence (267 bp, ON314264) obtained from the *D. marginatus* tick collected from patient 43/21 showed 99.63% identity with reported sequences of *R. raoultii* strains detected in *D. marginatus* tick species from the Crimean Peninsula (KU961541). Whilst *R. raoultii gltA* gene sequences (336 bp, ON314265) obtained from *D. reticulatus* tick species revealed high identities from 99.63% to 100% with sequences available in the GenBank, which were obtained from *R. raoultii* strains detected in *Hyalomma marginatum* and *Dermacentor silvarum* tick species from Portugal (LC229630) and China (KM386690, KF003009), respectively. In addition, the *gltA* gene sequences obtained from *R. helvetica* (266 bp, ON314267) and *R. monacensis* (two fragments, one of 369 bp, ON314268, and the other of 378 bp, ON314270) identified in *I. ricinus* ticks showed high identities 98,37% and 100% with sequences reported in *I. ricinus* tick species from Slovakia (MF673863) and Hungary (LC060717), respectively. Both *R. monacensis gltA* fragments identified in this study were 100% identical between them. Interestingly, *R. slovacica gltA* gene sequence (369 bp, ON314269) obtained from

Haemaphysalis spp. tick species shared 100% identity with a strain detected in *D. marginatus* tick species removed from a human patient with SENLAT syndrome in Italy (MT981150).

3.5. Phylogenetic analysis and genetic distance of SFGR strains

The *R. slovaca gltA* gene sequence obtained in this study formed a phylogenetic cluster with other *R. slovaca* strains identified in Italy, Slovakia, France, Uganda and China (Supplementary figure 1), suggesting that the *R. slovaca* strain identified in our study in *Haemaphysalis* ticks may circulate in different regions of the world. The *R. helvetica gltA* gene sequence formed a cluster with strains identified in other European countries including a strain identified in *I. ricinus* ticks from Slovakia, which concur with the blast identity analysis for this isolate described in the previous section (Supplementary figure 2). The two *R. monacensis gltA* gene sequences clustered together and they were obtained from *I. ricinus* ticks that attached to patients in the Fruška gora mountain, suggesting that close-related strains of this pathogen circulate in this location (Supplementary figure 3). The *R. raoultii gltA* gene identified in the *D. reticulatus* tick sample showed little genetic divergence from other isolates included in the tree (Supplementary figure 4). The phylogenetic tree built with the *R. raoultii ompB* gene sequence obtained from the the *D. marginatus* tick collected from patient 43/21 clustered with sequences from Ukraine, Germany and Russia (Supplementary figure 5), while the *R. helvetica ompB* gene from the capillary blood sample of the same patient clustered with a sequence previously identified in Serbia (Supplementary figure 6).

The genetic distances between the nucleotide sequences obtained in this study and sequences from other countries (excluding previous strains from Serbia) showed significant differences for *R. helvetica gltA* and *R. helvetica ompB* (Mann Whitney *U* test, $p < 0.05$, Figure 2), but no for *R. slovaca gltA*, *R. monacensis gltA*, *R. raoultii gltA* and *R. raoultii ompB* (Mann Whitney *U* test, $p > 0.05$, Figure 2). Particularly, the genetic distances between the *R. helvetica gltA*

and *R. helvetica ompB* obtained in this study were lower and higher than that from other countries, respectively. Concerning *R. monacensis gltA*, *R. helvetica gltA* and *R. helvetica ompB* for which sequences identified previously in Serbia were available, we found significant lower genetic distances for *R. monacensis gltA* obtained in this study (Mann Whitney *U* test, $p < 0.05$) and no difference for *R. helvetica gltA* and *R. helvetica ompB* (Mann Whitney *U* test, $p > 0.05$) (Figure 2).

3.6. One Health study revealed possible SFGR focus near a rural household in Begeč village

Since patient number 427/21 reported that tick acquiring location was her household, a field study was organised after she developed signs of SFGR infection. Five days after disease onset, a visit was organised to Begeč village (45.2411° N, 19.6221° E) where patient lived with her son, husband and two pet dogs West Highland White Terriers. Both dogs were examined for the presence of ticks and fleas, as well as all farm/pet animals (five dogs, one cat and two goats) in three nearby households. No ticks or fleas were detected on any animal, which is in accordance to statement of all owners that they routinely use repellents for the control of ticks and fleas. Blood samples were collected from the West Highland White Terriers dogs, while the son and husband were invited to Pasteur Institute Novi Sad for blood sampling and clinical examination. Further anamnesis revealed that patient's husband works as a forester in Nature park "Begečka Jama", from where he often returns to home with ticks attached to his uniform and patient recalled that often sees ticks on her husband's uniform after he returns from the work. Further examination revealed no clinical signs of SFGR disease in pet dogs of the family members and none of the collected blood samples were positive for SFGR DNA in the high-throughput microfluidic real-time PCR assay. However, the serological analysis revealed the presence of IgG antibodies reacting with *R. conorii* antigen in the sera sample collected from the patient's husband in 1:128 dilution.

4. Discussion

Although tick-borne rickettsioses have global distribution, majority of European continent is not considered to be heavily affected by these TBDs (Portillo et al., 2015). Currently, only Mediterranean area and south Europe are endemic for MSF that is caused by *R. conorii*. Researchers from many European countries, including Serbia, are reporting increasing number of ticks infected with pathogenic SFGR (Andersson et al., 2018; Guccione et al., 2021; Pascucci et al., 2021; Špitalská et al., 2021; Szekeres et al., 2016). Here we presented five cases of confirmed SFGR infection registered in a period of four months (Figure 1), which represent an increase compared to the incidence of these diseases in the previous two years 2019 (0/480; 0%) and 2020 (1/650; 0.15%). First suspected clinical SFGR infection in Serbia was described in 2020 (Banovic et al., 2021b), while in 2019 there was no recorded SFGR infection in examined patients within the same territory (Banovic et al., 2021a). None of the patients described here developed systemic signs and symptoms of SFGR infection, and all disease manifestations were local (i.e., on the site of previous tick attachment and within regional lymph nodes). Reason for milder forms of the diseases reported here may be early detection and fast administration of therapy after disease onset.

In contrast to the high prevalence of *R. helvetica* infection in ticks (Andersson et al., 2018; Szekeres et al., 2016) and frequent human exposure in endemic areas (Lindblom et al., 2016; Wolfel et al., 2017), the reports of clinical infection caused by this TBP in Europe are scarce. *Rickettsia helvetica* was reported as single causative agent in patients with acute febrile illness (Fournier et al., 2000), rash, myasthenia (Nilsson, 2009) and meningitis (Nilsson et al., 2010). *Rickettsia helvetica* infection in the tick feeding on human is found to be strongly associated with development of facial paralysis (Azagi et al., 2022). The patients described

here were registered with milder forms of *R. helvetica* infection, similar to previous cases of suspected *Rickettsia* infection registered in Serbian in 2020 (Banovic et al., 2021a), with development of non-expanding circular redness and eschar. However, it is noteworthy that patient 43/21 reported here with *R. helvetica* infection presented enlarged regional lymph nodes and elevated values of creatine kinase (CK) and lactate dehydrogenase (LDH) and developed a scar and discoloration at the place of previous eschar.

Rickettsia monacensis was detected as second most common SFGR in ticks removed from patients described here. This bacterium had not been reported previously as a human pathogen in Serbia. The reports of *R. monacensis* infection in Europe are mostly related to areas where MSF is endemic, causing spotted fever-like illness with eschar, but without maculopapular rash (Madeddu et al., 2016) or in the form of maculopapular rash without eschar, fever, headache and joint pain (Jado et al., 2007). In contrast, first and only case of *R. monacensis* infection in Croatia was detected accidentally together with *Borrelia afzelii* in erythema migrans biopsy (Tijssse-Klasen et al., 2013). The patients with probable *R. monacensis* infection in Serbia developed mild redness as the sign of the disease. One patient had induration at the lesion site, while other developed a papule at the lesion center with intense itching sensation. Systemic signs of infection and enlarged lymph nodes were registered only in one patient on the fifth day of doxycycline treatments, when he developed fever, nausea, and malaise.

Rickettsia raoultii and *R. slovaca* are most often reported as causative agents of clinical syndrome called SENLAT (Scalp Eschar and Neck Lymph Adenopathy after Tick bite) or TIBOLA (Tick-borne lymphadenopathy). The syndrome is considered to be most common tick-borne rickettsiosis in Europe (Oteo and Portillo, 2012), after MSF. TIBOLA cases caused by *R. slovaca* infections are described in Italy (Barlozzari et al., 2021), Greece (Kostopoulou et al., 2016), France (Mechai et al., 2009), Turkey (Emiroglu et al., 2021),

Russia (Igolkina et al., 2022; Sayfullin et al., 2021), Portugal (de Sousa et al., 2013) and Bulgaria (Komitova et al., 2003). Systemic manifestations of *R. raoultii* infection are most commonly fever, headache, weakness, and painful eschar as well as enlarged and painful lymph nodes (Igolkina et al., 2018). Patient can also develop leukopenia, thrombocytopenia, and elevated CRP and procalcitonin (Switaj et al., 2012). *Rickettsia raoultii* infection can be manifested with development of skin efflorescence (Földvári et al., 2013). Suspected *R. raoultii* case described in Serbian patient shares similarities with previously reported cases. In this 10-year-old boy, pain at tick attachment site occurred 5 days after *D. reticulatus* was removed but enlarged neck and retroauricular lymph nodes and eschar appeared only 4 days later (9 days after tick removal). He did not show any systemic signs of disease and all laboratory results were unremarkable.

Case of probable *Rickettsia slovaca* infection presented here is different from previously reported cases. Possible reason is that vector in this case is *Haemaphysalis* spp. leading to development of painful lesion with central ulceration on her left gluteus in addition to inguinal lymph node enlargement that were painful to palpation. Reason for these differences should be further examined, but there is a possibility that different manifestation can be linked with different tick vector species (*Dermacentor* vs. *Haemaphysalis*) and body region where *R. slovaca* is probably inoculated (head region vs. gluteus).

Emergence of diseases caused by SFGR are reported either due to objective increase of disease incidence or due to implementation of new diagnostic protocols that allow detection of diseases that were not recognisable before (Jakimovski et al., 2022; Kostopoulou et al., 2016). SFGR infection cases described in Serbian patients occurred during late spring and summer months. This distribution is in accordance with incidence of other SFG rickettsioses in continental parts of Balkans and it is possibly consequence of migration and more aggressive behaviour of ticks, as well as more frequent outdoor human activities (Baltadzhiev

and Popivanova, 2012; Banovic et al., 2021b; Jakimovski et al., 2022; Premaratna, 2022). Here we used combined sampling methods that allowed us to detect pathogens on biological material collected from the patient (i.e., direct diagnosis) and on the ticks removed from the same patients (i.e., xenodiagnostic). From the biological samples collected from the patients, capillary blood from tick attachment site/lesion center showed the highest sensitivity (4/9; 44.44%), while only one of 4 eschar crusts (1/4; 25%) was positive for genetic material of tested TBPs. Lesions caused by SFGR are consequence of vasculitis (i.e., inflammation of endothelial cells of blood vessels). Accordingly, capillary blood, containing traces of endothelial cells, is a sample of choice when aiming at direct detection of these intracellular bacteria (Biggs et al., 2016; Parola et al., 2013). In our study, detection of SFGR DNA in eschar crust samples was less successful compared to capillary blood. Similarly, Dubourg et al. (2014) reported low detection rate of *Rickettsia* spp. DNA in eschars collected from patients with SENLAT, as only 3 out of 9 analyzed eschar crusts were positive to these pathogens (Dubourg et al., 2014). In addition, Ibarra et al. (2006) had no positive findings from eschar samples of patients with DEBONEL/TIBOLA, despite SFGR *ompA* and/or *gltA* genes were detected in all tested ticks removed from patients and in approximately 30% of serum samples (Ibarra et al., 2006). Results of xenodiagnostic analyses in ticks removed from our patients are in accordance findings of Ibarra et al. (2006) since SFGR were detected in all tick infesting patients who developed suspicious clinical manifestations. Observed discrepancy between results from tick analysis and capillary blood/eschar crust analysis could be attenuated with inclusion of platelet fraction as analytic substrate, which could be more suitable for TBP detection (Banovic et al., 2022). One of limitations of our study is that we were not able to examine paired serum samples of patients described here. To partially overcome this limitation, we used a higher threshold to declare positive finding on IFA (1:64) as proposed by National Notifiable Diseases Surveillance System of Centers for Disease

Control and Prevention in reporting of probable or suspected SFGR infections (McQuiston et al., 2014).

Despite not being present in routine clinical application, xenodiagnostic methods applied here provided essential information about rickettsial ethology of observed manifestations. In addition, xenodiagnostic used here provided results of epidemiological value when combined with anamnestic data related to tick exposure site. Data provided here is suggesting existence of different SFGR foci on territory of Fruška Gora Mountain and 4 different settlements (of which 3 are within North Serbia). Supporting evidence for existence of SFGR foci on Fruška Gora Mountain was found during same year, when 10 additional SFGR-positive were ticks removed from the patients who identified location on Fruška Gora Mountain as tick exposure site (Banovic et al., 2022). In our opinion the occurrence and detection of five confirmed SFGR cases in Serbia is mainly consequence of TBP emergence, since the same approach in TBP and TBD detection was used in years 2019 and 2020, where zero and one SFGR case were detected, respectively. In addition, the number of tick-infested patients admitted during 2021 was much smaller than in 2020 (460 vs. 650) and similar as reported in 2019 (460 vs. 480). Currently, Serbia does not have any diagnostic guidelines, case definition or surveillance program related to SFGR infection in humans. In addition, majority of medical laboratories do not have capacities to confirm or detect tick-borne rickettsial diseases.

5. Conclusions

Here we reported nine clinical cases of atypical TBDs in Serbian patients. In all cases where xenodiagnostic was available, SFGR DNA was detected in ticks attached to the skin of the patients, which suggests probable pathogen transmission and incriminates the identified ticks as potential disease vectors. Based on the evidence reported here we conclude that we have

registered five clinical cases with SFGR infection (i.e., 43/21, 197/21, 401/21, 212/21, 140/21), 2 probable cases of SFGR infection with positive serology, positive xenodiagnostic PCR finding and negative diagnostic PCR finding (i.e., 296/21 and 427/21) and 2 suspected cases of SFGR infection with compatible clinical manifestation and tick exposure with positive xenodiagnostic PCR finding, but with no confirmation by diagnostic assays (i.e., 106/21 and 45/21). Examination of household members of patient with probable SFGR infection organised following One Health tenets revealed Nature Park "Begečka Jama" as possible SFGR foci. Phylogenetic and genetic analysis of the *R. helvetica ompB* sequence identified in patient 43/21 suggests intra-species sequence diversity in *R. helvetica* strains infecting humans in Serbia. In addition, here we described first clinical cases of infection caused by *R. helvetica* in Serbian population with emphasis on their atypical manifestation. Sustained surveillance and monitoring of tick-borne diseases in Serbia will create awareness on the risk of these zoonotic diseases for human health in the country.

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7. Conflict of interest statement

The authors declare no conflict of interest.

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Figure legends

Figure 1. Skin lesions in representative SFGR cases. (A) Patient 43/21; black arrows point to redness surrounding eschar that is developed at the place of previous tick bite. (B) Patient 106/21; black arrows point to edematous redness surrounding eschar that is developed at the place of previous tick bite. (C) Patient 212/21; black arrows point to discrete redness surrounding epithelial defect at the place of tick attachment. (D) Patient 140/21; black arrows point eschar developed at the place of previous tick infection.

Figure 2. Genetic diversity of SFGR strains identified in Serbia. The genetic distances between the *R. slovaca gltA* (Supplementary figure 1), *R. helvetica gltA* (Supplementary figure 2), *R. monacensis gltA* (Supplementary figure 3), *R. raoultii gltA* (Supplementary figure 4), *R. raoultii ompB* (Supplementary figure 5) and *R. helvetica ompB* (Supplementary figure 6) nucleotide sequences included in each phylogenetic tree were calculated as p-distances. Three sets of pairwise p-distance values were collected and compared. P-distance values resulting from comparisons between sequences identified in this study and others included in each tree (excluding sequences previously identified in Serbia) are labelled as '1'. P-distance values resulting from comparisons between sequences previously identified in Serbia and others included in the trees (excluding sequences identified in this study) are labelled as '2'. P-distance values resulting from comparisons between all sequences in the tree (excluding sequences obtained in Serbia, in this or previous studies) are labelled as '3'. The statistical differences between p-distances in '1', '2' and '3' were assessed using the Mann Whitney *U* test (* $p < 0.05$, ns—not significant).

LDH	Increased	WNL	WNL	WNL	WNL	WNL	WNL	WNL	WNL
CK	Increased	WNL	WNL	Increased	WNL	WNL	WNL	WNL	WNL
Time from tick bite to serum sampling	4 weeks	-	4 weeks	-	4 weeks	4 weeks	4 weeks	4 weeks	4 weeks
Anti-SFGR IgG (Maximum serum dilution reactive in the IFA)	Positive (1:256)	PNA	Negative	PNA	Positive (1:256)	Negative (1:32)	Positive (1:128)	Positive (1:128)	Positive (1:128)
Outcome of antibiotic therapy	Patient recovery	Patient recovery	Patient recovery	Patient recovery	Patient recovery	Patient recovery	Patient recovery	Patient recovery	Patient recovery
Detection of <i>Rickettsia</i> spp. in clinical and tick samples									
Type of sample	Microfluidic PCR results (accession number of sequenced gene fragments)								
Eschar crust	Negative	Negative	Negative	<i>Rickettsia</i> sp.	NA	NA	NA	NA	NA
Capillary blood	<i>R. helvetica</i> (<i>ompB</i> , ON314263)	Negative	Negative	Negative	<i>R. helvetica</i>	<i>Rickettsia</i> sp.	Negative	<i>Rickettsia</i> sp.	Negative
Tick	<i>R. raoultii</i> (<i>ompB</i> , ON314264)	<i>R. raoultii</i> (<i>gltA</i> , ON314265)	<i>R. helvetica</i>	-	<i>R. helvetica</i> (<i>gltA</i> , ON314267)	<i>R. helvetica</i>	<i>R. monacensis</i> (<i>gltA</i> , ON314268)	<i>R. monacensis</i> (<i>gltA</i> , ON314270)	<i>R. slovaca</i> (<i>gltA</i> , ON314269)

2 # Information provided by patient.

3 ## Feeding time estimated from coxal and scutal indices.

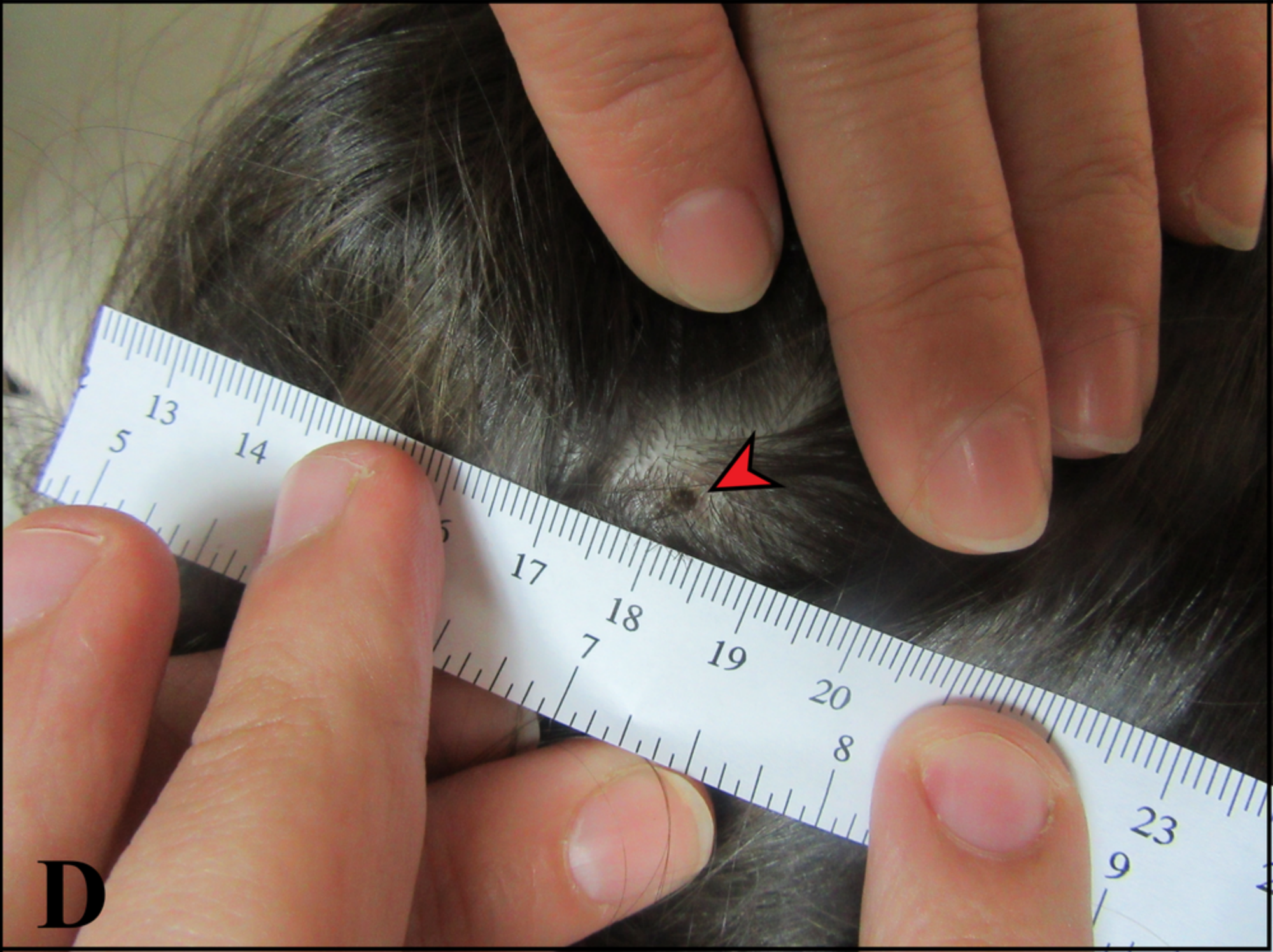
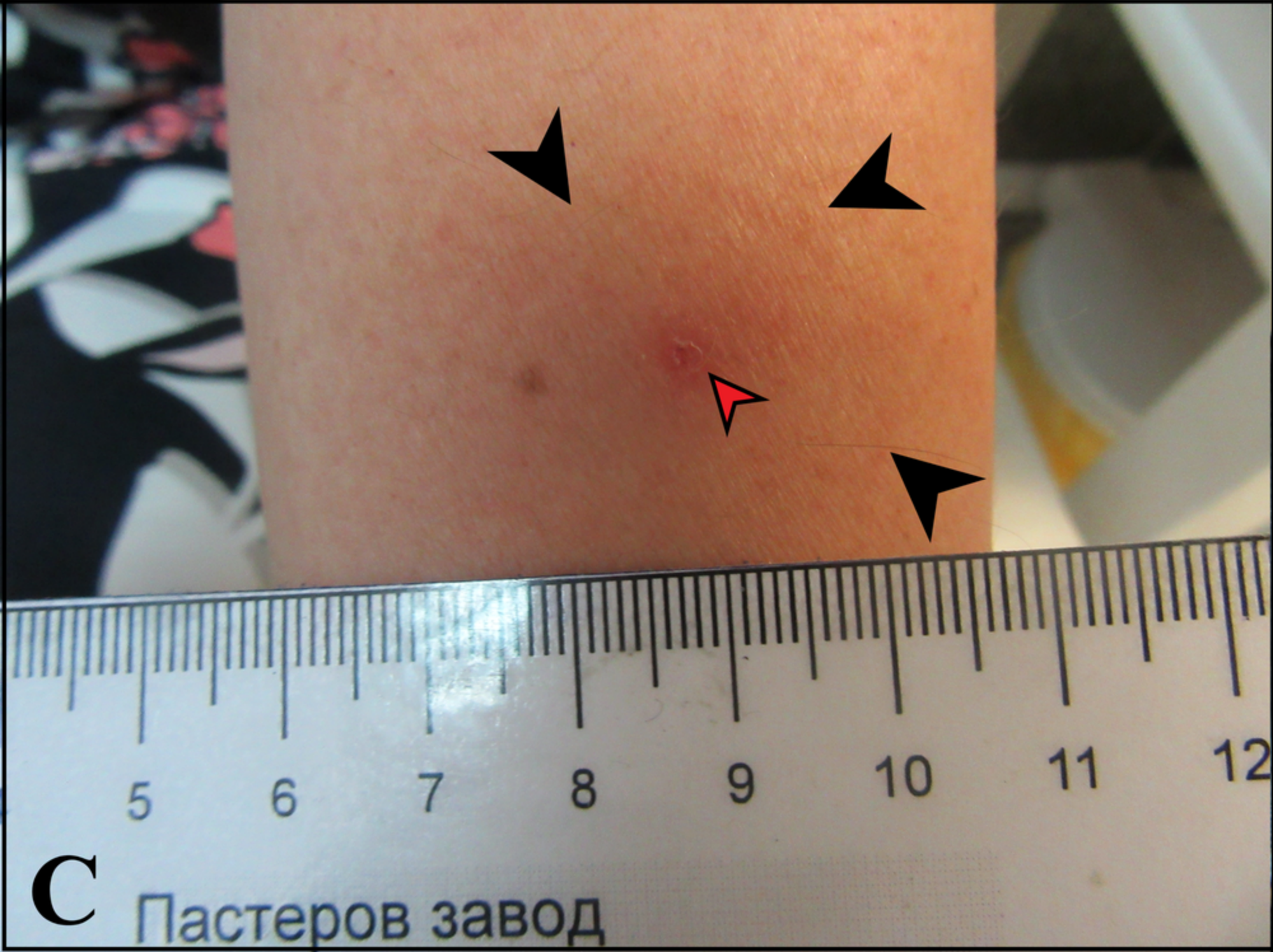
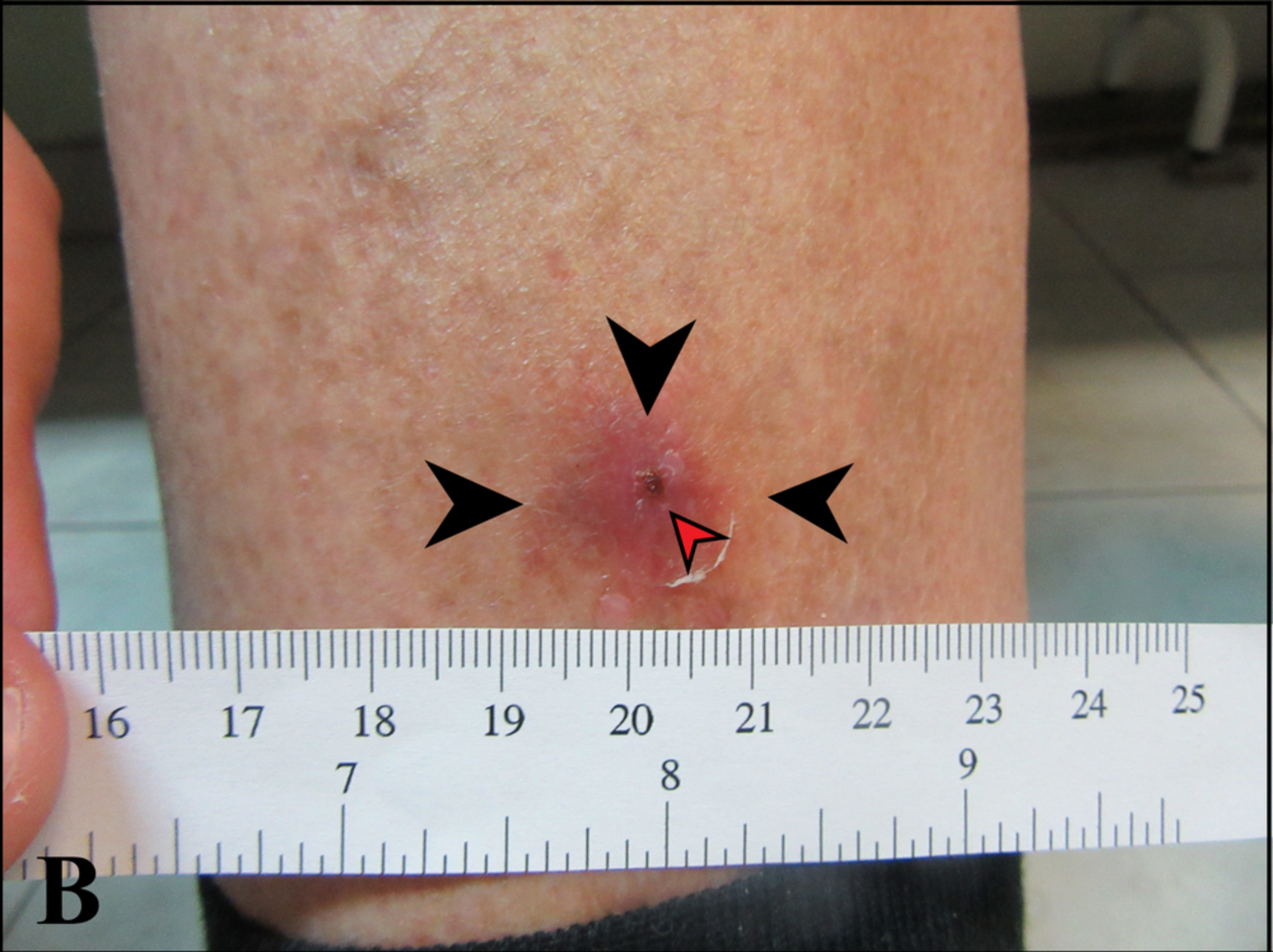
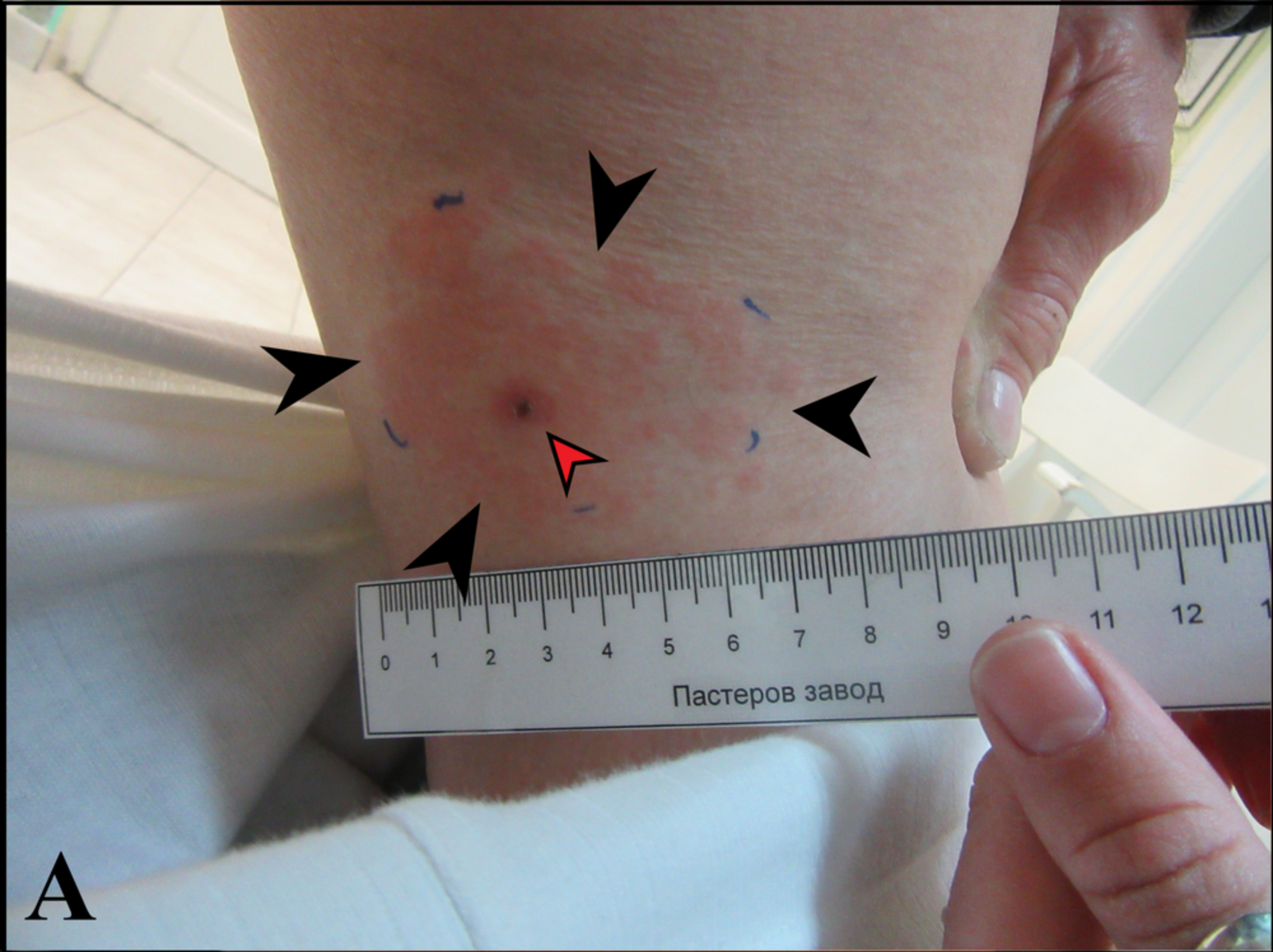
4 *Abbreviations are as follow: WBC (White blood cells), RBC (Red blood cells), HCT (Hematocrit), HGB (Hemoglobin), PLT (Platelets), AST (Aspartate transaminase),

5 ALT (Alanine transaminase), GGT (Gamma-glutamyl transferase), CRP (C-reactive protein), LDH (Lactate dehydrogenase), and CK (Creatine kinase).

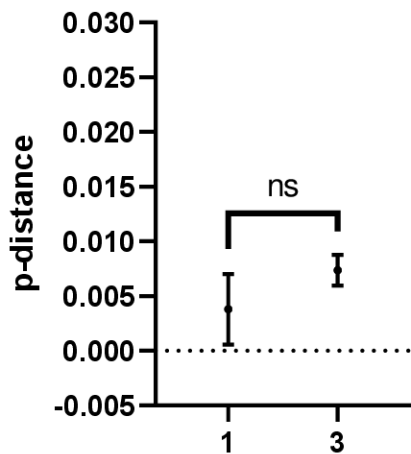
6 WNL (Values within normal limits).

7 PNA (Patient not available for sample collection).

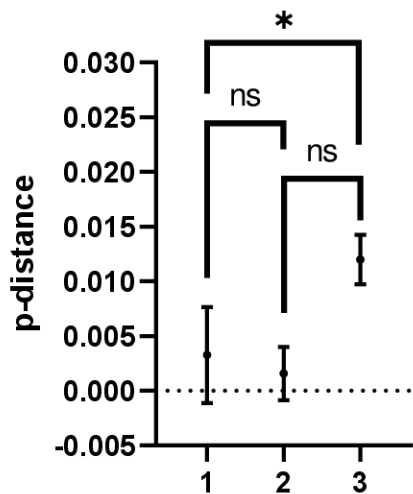
8 NA (Not available, as eschar was absent).



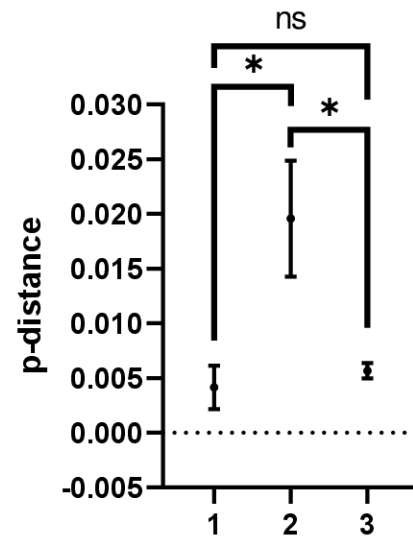
Rickettsia slovaca gltA



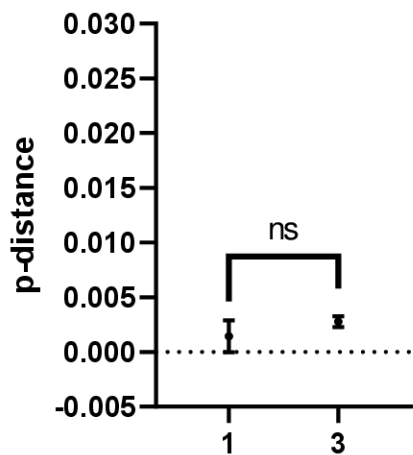
Rickettsia helvetica gltA



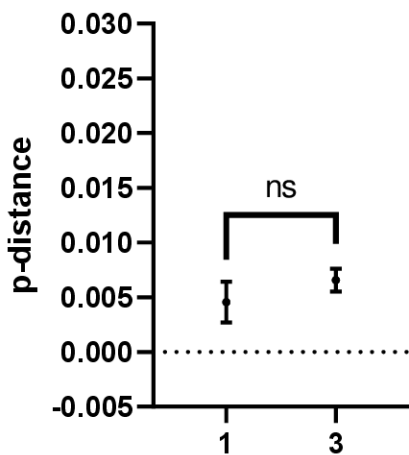
Rickettsia monacensis gltA



Rickettsia raoultii gltA



Rickettsia raoultii ompB



Rickettsia helvetica ompB

