FIRST MOLECULAR IDENTIFICATION OF *RICKETTSIA CONORII* SUBSPECIES *CONORII* AS ETIOLOGIC AGENT OF MEDITERRANEAN SPOTTED FEVER IN SOUTHEASTERN ROMANIA

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ABSTRACT

Introduction: Mediterranean spotted fever (MSF) is a spotted fever group (SFG) rickettsiosis caused by *Rickettsia conorii* endemic in Mediterranean countries as well as in countries around the Black Sea. In Southeastern Romania MSF is endemic, with an overall incidence of 4.2 per 100,000 population, established for 2016 by the National Center for Disease Prevention and Control. Diagnosis of MSF is complex, based on clinical, epidemiological and laboratory data.

Objectives: The aim of the study is to describe the usefulness of molecular tests in MSF diagnosis, as well as to provide the first molecular characterization of *R. conorii* subspecies *conorii* responsible for MSF in Romania.

Methods: Molecular biology assays (nested PCR) were used for testing serum samples collected from 20 patients with MSF serologically confirmed.

Results: Molecular diagnosis of MSF was performed by nested PCR assays on *rompA* and *rompB* genes, and was confirmed by sequencing of obtained amplicons. Molecular analysis based on a *rompA* gene fragment allowed placing the isolate detected in the sera collected from a Romanian patient with fatal MSF in the same cluster with other *R. conorii* subspecies *conorii* isolates.

Conclusions: We present the first data on the use of molecular tools for the early diagnosis of MSF, as well on molecular identification of *R. conorii* subspecies responsible for MSF in five counties in Southeastern Romania.

Keywords: Mediterranean spotted fever, *Rickettsia conorii*, molecular diagnosis, tick-borne rickettsioses.

REZUMAT

Introducere: Febra pătată mediteraneană (*Mediterranean spotted fever* - MSF) sau febra butonoasă (FB) este o rickettsioză din grupul febrelor pătate (*spotted fever group* - SFG) determinată de *Rickettsia conorii,* endemică în țările bazinului mediteranean, precum și în cele cu ieșire la Marea Neagră.

Febra butonoasă este endemică în sud-estul României, înregistrând în anul 2016 o incidență totală de 4,2/100 000 locuitori, conform datelor de supraveghere transmise de către Centrul Național de Supraveghere și Control al Bolilor Transmisibile (CNSCBT).

Obiective: Scopul acestui articol este de a descrie utilitatea testelor de biologie moleculară în diagnosticul FB, precum și de a furniza prima caracterizare moleculară a subspeciei de *Rickettsia conorii* responsabilă de FB în România.

Metode: Au fost utilizate metode de biologie moleculară (nested PCR) pentru testarea probelor de ser provenite de la 20 pacienți cu FB confirmată prin teste serologice.

Rezultate: Diagnosticul molecular de FB a fost efectuat prin testele nested-PCR pe 2 gene țintă, *rompA* și *rompB* și a fost confirmat prin secvențierea ampliconilor obținuți. Analiza moleculară realizată pe fragmentul genei *rompA* a permis încadrarea tulpinii, recoltate de pacientul cu FB fatală, în același cluster cu alte tulpini de *R. conorii* subspecia *conorii.*

Concluzii: Articolul prezintă primele date privind utilizarea testelor de biologie moleculară în diagnosticul precoce al FB, precum și prima identificare moleculară a subspeciei de *Rickettsia conorii* responsabilă de cazurile de FB din cinci județe din sud-estul României.

Cuvinte-cheie: febra pătată mediteraneană, *Rickettsia conorii*, diagnostic molecular, rickettsioze transmise de căpușe.

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INTRODUCTION

Mediterranean spotted fever (MSF) is a tick-borne rickettsiosis caused by *Rickettsia conorii*, with a large distribution on the European continent, being present in the Mediterranean countries as well as in countries around the Black Sea [1-3].

R. conorii subsp. *conorii* is the main etiological agent of MSF in Europe, followed by *R. conorii* subsp. *israelensis* (Portugal and Italy), *R. conorii* subsp. *caspia* (Astrakhan, Kosovo and France), and *R. conorii* subsp. *indica* (Spain) [1, 4-5].

MSF has been a notifiable disease in Romania since 2000, and was until recently the only human tick-borne rickettsiosis known to clinicians [6]. MSF is endemic in Southeastern Romania, with an overall incidence of 4.2 per 100,000 population, as established for 2016 by the National Center for Surveillance and Control of Communicable Diseases [7]. The highest incidence was recorded in Tulcea county (15.76/ 100,000 inhabitants), followed by Constanta county (5.73/ 100,000 inhabitants) [7].

The vector and reservoir of *R. conorii conorii* is the brown dog tick, *Rhipicephalus sanguineus*.

In the last years, two molecular studies conducted in distinct tick populations from different areas of Romania identified the presence of four rickettsial pathogens: *R. monacensis* and *R. helvetica* in *Ixodes ricinus* [6, 8], and *R. slovaca* and *R. raoultii* in *Dermacentor marginatus,* respectively [6].

R. conorii was identified as the main etiologic agent responsible for MSF in Romania, although hospitalized patients having clinical picture of tick bite and secondary skin eschar suggestive for SENLAT syndrome (scalp eschar and neck lymphadenopathy after a tick bite) were described. SENLAT syndrome was formerly known as TIBOLA (tick- borne lymphadenopathy) or DEBONEL (Dermacentor-borne necrotic erythema and lymphadenopathy), and is caused by several bacteria, mostly rickettsial agents such as *R. raoultii* and *R. slovaca* [9, 10]. Two cases of infection with *R. massiliae*, agent of spotted fever disease (MSF), one case with *R. slovaca*, and one case with *R. slovaca*/*R. raoultii* presenting to the Hospital for Infectious and Tropical Diseases, Bucharest, Romania, were serologically diagnosed [10].

Usually MSF is a benign disease, characterized by skin rash, high fever, and a characteristic ulcer at the tick bite site called "tache noir". In 6 to 10% of MSF cases severe complications may occur, including multiorgan involvement (hepatic, renal, cardiac, neurological), often resulting from delayed diagnosis [11]. MSF complications are more common in immunocompromised patients (so-called malignant form of MSF) [12].

The aim of the study was to introduce a molecular assay for rapid diagnosis of MSF cases in the practice of the Reference Laboratory for Vector-borne Infections in Cantacuzino Institute, as well as to identify at molecular level the subspecies of *R. conorii* responsible for MSF cases in Southeastern Romania.

MATERIALS AND METHODS

Suspect for MSF patients, residents in Bucharest and five counties in Southeastern Romania (Prahova, Tulcea, Călărași, Buzău and Constanța), were recruited during the transmission season (May–October 2010) for laboratory testing. Twenty patients with laboratory confirmed MSF produced by *R. conorii* were selected for molecular testing. Serum samples of patients have been tested using Indirect Immunofluorescent Assay (IFA) kit (Vircell, Spain) for detection of IgG antibodies against *R. conorii*, in an acute serum sample collected at least 7-10 days after onset of symptoms. Also, a second (convalescent) serum sample collected at 10-14 days after the first sample was tested to determine the seroconversion (for cases with the first serum negative for IgG) or the fourfold increase of antibodies titer. In one patient with a suspicion of central nervous system (CNS) infection, a CSF sample was also available. For molecular testing were selected only the serum samples collected from MSF serologically confirmed patient-cases at maximum 14 days post-onset of symptoms.

Genomic DNA was extracted from serum and CSF samples using Pure Link, Genomic DNA kit (Invitrogen). For *R. conorii* genome

detection in clinical samples we performed two nested PCR assays based on specific primers derived from the rickettsial outer membrane proteins B and A genes (*rompB* and *rompA*) of *R. conorii*. The assay for *rompB* gene amplification was adapted after the nested PCR protocol described by Choi in 2005 [13], whereas for *rompA* gene amplification we adapted the protocol described by Sardelic *et al*. in 2003 [14]. Thus, in the first round of amplification we used ompA 190-70 and ompA 190-701 primers, which yielded an amplification product of 632 bp, whereas in the second round the primers SLO 1F and SUI 1R amplified an internal fragment of the amplicon obtained in the first round, producing an amplicon of 495 bp.

All the amplification products were checked by 1.5% agarose gel electrophoresis in TAE 1X buffer. The amplicons for *rompA* gene were directly sequenced in an automatic sequencer (3100-Avant Genetic Analyzer, Applied Biosystems) using BigDyeV3.1 kit.

The obtained *rompA* sequences were compared with similar sequences available in GenBank by using BLAST. Phylogenetic analysis was conducted with Mega 6 software (Maximum Likelihood method, 1000 bootstrap replicates) on a 423 nucleotide fragment spanning *rompA* gene.

RESULTS

Molecular tests were performed in serologically *R. conorii* MSF confirmed cases which were recorded during the transmission season (May-October), in the following counties: Prahova (2), Tulcea (5), Constanța (6), Buzău (1), Călărași (2), and Bucharest city (5).

The nested PCR assay on *rompB* gene allowed detection of *R. conorii* DNA in 21 analyzed serum samples collected in the first 7 to 14 days after onset, whereas the nested-PCR on *rompA* gene allowed detection in only 12 samples. For both target genes, the specific amplicons were obtained during the second round of PCR assays.

Molecular diagnosis of MSF cases performed by nested-PCR assays on *rompB* and *rompA* genes was confirmed by sequencing of the obtained amplicons. Molecular analysis based on a *rompA* gene fragment placed the isolate detected in the sera collected from a Romanian patient with MSF in the same cluster

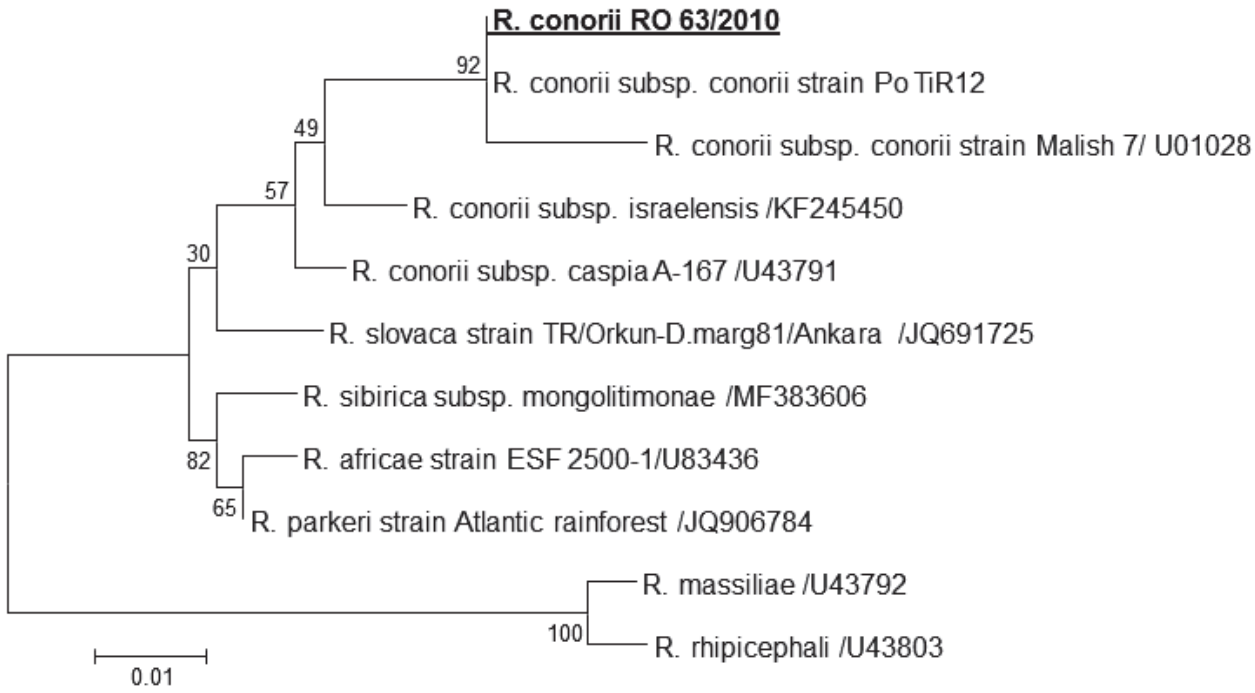

Fig. 1 - Dendrogram based on a 423 nt. *romp*A gene fragment depicting the phylogenetic relatedness between several Rickettsia spp. and Romanian *R. conorii* strain

with other *R. conorii* subsp. *conorii* isolates (Fig. 1). The Romanian sequence of *rompA* gene obtained from the fatal case of MSF used in Fig. 1 was named *R. conorii* RO 63/2010 and was deposited in the GenBank, receiving the accession number MH349045.

One peculiar case was a fatal case from Medgidia (Constanța county): a 58 year old male, with underlying oncological disease, who presented with high fever (40-41°C), petechial and erythematous rash, meningitis, thrombocytopenia (72.000/mm^3).

As he was initially suspected of non-infectious hyperthermia caused by the very hot weather, the samples were taken late in the course of the disease. Molecular tests were performed for both serum and CSF samples of this patient, and both were *rompA* and *rompB* PCR positive and confirmed by *rompA* sequencing. To the best of our knowledge this is the first report of a fatal case of MSF in Romania in which both serum and CSF were PCR positive.

DISCUSSION

The selection in the present study of the target genes for molecular diagnosis of serologically confirmed MSF cases was driven by the fact that previous studies have shown that *rompB* gene is a good target for high sensitivity molecular screening of spotted fever group (SFG) rickettsiae, in different types of clinical samples, as well as in ticks. On the other hand, *rompA* gene is more specific than *rompB* gene, being an appropriate gene target for discrimination among *R. conorii* subspecies.

Our molecular results are in accordance with previous studies that have shown that PCR-based amplification methods are a useful diagnostic tool of MSF in the early phase of the illness, when antibodies are not detectable [15]. PCR based on *rompB* proved to be a sensitive screening test of serum samples from MSF acute cases, while *rompA* PCR followed by sequencing was useful for the identification of *R. conorii* subspecies involved in human pathogenesis. Early diagnosis of MSF which may be performed by PCR is critical to an adequate management of the patients. In some cases, the course of the disease is severe, and early implementation of specific antibiotherapy is life saving.

The phylogenetic analyses of all 12 *rompA* sequences showed that *R. conorii* strain responsible for the MSF cases diagnosed in this study is closely related to *R. conorii* subsp. *conorii* strain Malish 7, accession number AE006914.

Data on the diversity of SFG rickettsiae, other than *R. conorii conorii*, responsible for human infections in Romania, are scarce: in tick samples from different areas of Romania of four human pathogenic rickettsiae, *R. monacensis* and *R. helvetica* in *Ixodes ricinus* [6, 8] *R. slovaca* and *R. raoultii* in *Dermacentor marginatus* [6] were identified. TIBOLA cases determined by *R. slovaca* and *R. raoultii* as well as MSF cases determined by *R. massiliae* were reported recently [10].

Climate change has led to a northward expansion of several tick species and to an increase in the aggressiveness of the brown dog tick, resulting in increased incidence of *Rh. sanguineus*-transmitted pathogens (*R. conorii*, *R. rickettsii*, *R. massiliae*) [16, 17]. In the current context of increasing international travel and expansion of the areal of vector ticks, the number of both autochthonous and imported cases of SFG rickettsioses may be increasing. Therefore the introduction of molecular detection and molecular identification of pathogenic rickettsiae in the practice of the reference laboratory is of paramount importance. The PCR detection followed by sequencing remain, for many laboratories, the only diagnostic tools for *Rickettsia* species in which commercial kits for serologic diagnostic are not available.

In conclusion, we are reporting, for the first time in Romania the molecular diagnosis of MSF cases, including of a fatal case, and the identification of *R. conorii conorii*, responsible for 20 MSF cases from five counties in the southeast of the country, as shown by the 99 % similarity to the type isolate.

Conflict of interests: None to declare.

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