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VALIDATION OF A DUPLEX REAL-TIME PCR ASSAY FOR THE MICROBIOLOGICAL DIAGNOSIS OF RICKETTSIA spp. INFECTION IN THE METROPOLITAN AREA OF BOLOGNA

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INTRODUCTION

Rickettsioses are emerging arthropod-borne zoonoses (fleas, ticks, lice and mites) caused by gram-negative, intracellular obligate bacteria, of the genus *Rickettsia*. The latter is divided into two main groups: Typhus Group (TG - *R. prowazekii and R. typhi*) and Spotted Fever Group (SFG - *R. conorii, R. africae, R. rickettsii, R. akari and R. slovaca*). According to European surveillance data (European Centre for Disease Prevention and Control, ECDC) collected between 2000 and 2009, Italy together with Portugal and Spain, shows the most cases of rickettsiosis (4,609, 2,837, 651 cases respectively). The results of surveys on the seroprevalence of anti-*Rickettsia* spp. antibodies in humans showed a difference, with a variability of 4% to 37%, between Northern and Southern Italy (Figure 1). The clinical signs of the disease may be hyperpyrexia, arthralgia, lymphadenopathy, maculo-papular exanthema and eschar, also known as tache noire, an ulcer-necrotic area at the site of pathogen inoculation. Treatment involves doxycycline as the first choice, but azithromycin and clarithromycin may be used if the disease is severe. Microbiological diagnosis is essentially based on serological investigations, but to reduce the diagnostic time it may be useful to combine molecular tests using Real-time PCR (RT-PCR).

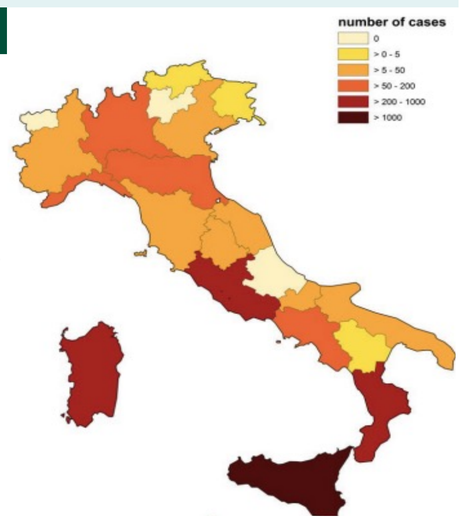

Figure 1. Distribution of the number of *Rickettsia* cases in Italy in the years 2000-2009

METHODS

At the Laboratory of Infectious Serology of the Operative Unit of Microbiology, Azienda Ospedaliero-Universitaria di Bologna (IRCCS), in addition to searching for species-specific IgG (*Rickettsia conorii* VIRCLIA IgG MONOTEST, Vircell), an in-house RT-PCR was developed. The primers and probes were designed to amplify both a variable 149 base-pair portion of the 16S rRNA gene as it is common to the different *Rickettsia* species belonging to the SFG group, and the citrate synthase gene (gltA) which, being less conserved among *Rickettsia* spp, was chosen to detect species belonging to the TG group.

RESULTS

From January 2023 to September 2024, anti-*Rickettsia conorii* IgG was detected in serum samples from 392 patients in the Bologna Metropolitan Area. In particular, 42/392 (10.7%) patients were positive for serological investigation and 9 of these (21.4%) reported compatible symptoms (**Table 1**); 8/9 presented an eschar (88.8%). Below are the results for 7 of these 8 patients for whom duplex RT-PCR was performed on both the eschar and the underlying vesicle fluid: 3 (42.8%) were negative for both targets, 2 (28.6%) were positive for the gene for SFG in both samples while for 2 (28.6%) amplification for the target for SFG was observed in only one material. Finally, in only one case was only vesicle fluid taken and the result of the RT-PCR test was also positive for the SFG gene (**Table 2**).

	CLINICAL SIGNS
BO_01	Hyperpyrexia; lymphadenopathy; maculo-papular rash on palms and trunk
BO_02	Hyperpyrexia up to 40°C; maculo-papular rash over the whole body
BO_03	Hyperpyrexia; arthralgia; maculo-papular rash on trunk, limbs, palms of hands and soles of feet
BO_04	Hyperpyrexia; headache; maculo-papular rash on trunk, limbs, palms of hands and soles of feet
BO_05	Arthralgia; lymphadenopathy; headache
BO_06	Hyperpyrexia up to 40°C; lymphadenopathy; maculo-papular rash on lower limbs
BO_07	Arthralgia; lymphadenopathy; headache
BO_08	Mild hyperpyrexia; maculo-papular rash on lower limbs
BO_09	Hyperpyrexia up to 40°C; arthralgia; maculo-papular rash on right arm

Table 1. Clinical signs of the 9/42 (21.4%) patients with positive serology. All, except BO_01, presented with eschar.

	RT-PCR ON VESICLE FLUID	RT-PCR ON ESCHAR
BO_01	POSITIVE	NOT TESTED
BO_02	POSITIVE	POSITIVE
BO_03	NEGATIVE	POSITIVE
BO_04	NEGATIVE	NEGATIVE
BO_06	NEGATIVE	NEGATIVE
BO_07	NEGATIVE	NEGATIVE
BO_08	POSITIVE	NEGATIVE
BO_09	POSITIVE	POSITIVE

Table 2. Results of RT-PCR performed on vesicle and/or eschar swabs

CRITERIA	SCORE*
Epidemiological criteria	
Originating from endemic areas	2
Clinical manifestations in the period from April to October	2
Contact (possible or certain) with animal ticks	2
Clinical criteria	
Hyperpyrexia > 39°C	5
Eschar	5
Maculo-papular rash	5
At least two of the above criteria	3
All three of the above criteria	5
Laboratory evidence	
Platelets < 150 10^9/L	1
Transaminases > 50 U/L	1
Bacteriological criteria	
Positive blood cultures for *Rickettsia conorii*	25
Presence of *Rickettsia conorii* in biopsy samples	25
Serological criteria	
IgG > 1/128	5
IgG > 1/128 e IgM > 1/64	10
Antibody titre quadrupled 15 days after first collection	20

*Score > 25: positive diagnosis for infection with *Rickettsia* spp.

Table 3. ESCMID, diagnostic criteria for *Rickettsia* spp. infection.

CASE BO_02

- **Patient admitted to the Emergency Department of the Budrio Hospital (Eastern Plain) for:**
  - **hyperpyrexia with temperature up to 40°C;**
  - **after 3 days from the onset of fever the patient presented maculo-papular exanthema to the entire body;**
  - **on the right thigh there was a lesion compatible with tache noire which appeared after the onset of the rash.**
- **RT-PCR positive for SFG target for both eschar and underlying vesicle fluid;**
- **Patient admitted with a diagnosis of rickettsiosis following a tick bite;**
- **During hospitalisation, anti-*Rickettsia conorii* IgG antibodies were tested and found to be positive;**
- **Doxycycline 100 mg every 12 hours was administered during the hospitalisation with excellent clinical and laboratory response;**
- **At discharge, a serological check-up was recommended approximately 15 days after the first sampling as indicated by the ESCMID (European Society of Microbiology and Infectious Diseases) guidelines (Figure 2) (Table 3).**

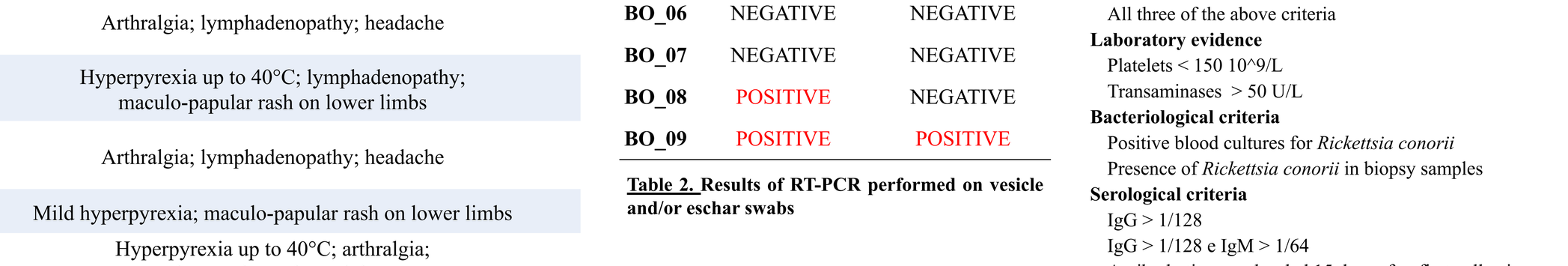

Figure 2. Timeline of clinical and diagnostic events of case BO_02

CONCLUSIONS

Despite the limited number of samples analysed, the introduction of the duplex RT-PCR test has proved useful in the early detection of cases with a strong clinical suspicion. In fact, the use of serological tests alone poses a limitation in diagnosis as they are unable to identify infection in the acute phase. The positive experience has made it possible to define, in collaboration with the OU of Infectious Diseases, a management protocol for suspected cases from all the districts of the Bologna Metropolitan Area. However, the pan-*Rickettsia* RT-PCR test is not able to discriminate the different *Rickettsia* species belonging to the two main groups, therefore our future goal is to be able to achieve species identification through gene sequencing.