Detection of Rickettsia in Rhipicephalus sanguineus Ticks and Ctenocephalides felis Fleas from Southeastern Tunisia by Reverse Line Blot Assay

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Ticks (n = 663) and fleas (n = 470) collected from domestic animals from southeastern Tunisia were screened for Rickettsia infection using reverse line blot assay. Evidence of spotted fever group Rickettsia was obtained. We detected Rickettsia felis in fleas, Rickettsia massiliae Bar 29 and the Rickettsia conorii Israeli spotted fever strain in ticks, and Rickettsia conorii subsp. conorii and Rickettsia spp. in both arthropods. The sensitivity of the adopted technique allowed the identification of a new association between fleas and R. conorii subsp. conorii species. The presence of these vector-borne Rickettsia infections should be considered when diagnosing this disease in humans in Tunisia.

Climate and land-use changes as well as sociodemographic and technological evolution are affecting many biological systems and influencing the emergence and spread of infectious diseases. This has led to an interest in zoonotic vector-borne diseases, including rickettsioses. These emerging infections appear when the obligate intracellular bacteria of the Rickettsia genus infect the endothelial cells. They are transmitted to humans and other animals by arthropod bites and are endemic in several regions where they continue to be a health problem with many human cases registered every year. The rickettsial species were widely believed to be restricted to their specific vectors and thus limited to specific areas. However, the detection of many Rickettsia species in different vectors in several areas worldwide has changed this assumption (1).

Species in the Rickettsia genus are divided into two groups: the spotted fever group (SFG) and the typhus group (TG). These illnesses are caused by about 22 species. Mediterranean spotted fever (MSF) is caused by Rickettsia conorii and is a disease described for the first time in Tunisia (2) and the most frequently observed spotted fever infection recorded in public hospitals. However, many clinical, serological, and molecular studies have demonstrated the presence of R. conorii, Rickettsia felis, and Rickettsia typhi in patients (3, 4, 5, 6). A high seroprevalence was also detected among Tunisian blood donors: 8% tested by Western blotting showed the characteristic profile of R. conorii (3). Despite the longstanding presence of the rickettsiosis and its medical importance in Tunisia, the arthropod vectors have been poorly investigated.

The detection and isolation of Rickettsia sp. require specialized laboratories with a high degree of expertise, primarily because of the species’ intracellular life cycle. For this reason, the diagnosis of Rickettsia infection is commonly based on clinical human descriptions and serological analyses (5). Several useful and more sensitive molecular tools such as real-time PCR and reverse line blot (RLB) assay have been currently developed to simultaneously detect and identify Rickettsia species in hosts and vectors (7).

While serological studies have demonstrated that various Rickettsia species circulate in patients in Tunisia, almost no data are available on the potential arthropod vectors. This study was conducted in southeastern Tunisia to identify Rickettsia spp. in arthropods (ticks and fleas) likely to be potential vectors of human rickettsial disease, using RLB assay confirmed by sequencing.

MATERIALS AND METHODS

Study. Our study was conducted in the Sfax governorate in southeastern Tunisia, with a population of 1 million inhabitants, or 8.5% of the total Tunisian population. This region, which plays a major role in the Tunisian economy, is situated in the arid bioclimatic zone with the lowest annual rainfall rate (200 mm/year). Agriculture focuses primarily on the cultivation of olive trees. Livestock production is also important, with an estimated 860,000 head of sheep, goats, and cattle in the region’s 15 municipalities.

Collection and identification of ticks and fleas. Ticks and fleas, feeding on domestic animals (dogs, sheep, and goats), were manually removed from July to October 2009 in eight municipalities (Sfax Sud, Sfax Ouest, Sakiet Ezzit, Sakiet Eddayer, Agareb, Jebeniana, Malloulech, and Karkenah). All collected specimens were identified to species level using appropriate taxonomic keys (8, 9). The map showing the distribution of arthropods collected from the different municipalities was constructed using ArcGIS 9 (version 9.3.1) software.

DNA extraction and PCR amplification. DNA was extracted from ticks and fleas using the QIAamp tissue DNA kit (Qiagen, Hilden, Germany), preceded by a 3-h treatment with a protease K solution (20 mg/ml). Water was included as a negative control to every 20 samples to test for possible contamination. The DNA concentration was determined with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). DNA from each sample between 50 and 400 ng was subjected to PCR assays and RLB hybridization to detect Rickettsia spp.

Amplification of rickettsial DNA was performed using primers spanning the 23S-5S intergenic region (forward primer RLBl-23S-5′-TGTGATAGGTCRGRGTGTCGAAGCAG-3′ and reverse primer RLBl-23S-5′-TCGGGAYGGGATCGTGTGTTTC-3′), producing ampli-
cons of approximately 330 to 533 bp in length (7). PCRs were performed in a thermocycler (PerkinElmer 2400). DNA amplification was done in a 25-μl reaction volume with 5 mM Tris-HCl-Cl₂Mg, 200 mM each deoxy-
nucleoside triphosphate, 0.75 U of Taq TaKara DNA polymerase (Applied Biosystems, Branchburg, NJ), with primers used at a final concentration of 5 pmol followed by the addition of 5 μl of arthropod DNA extract. PCR
cycling included an initial denaturing step of 15 min at 94°C, followed by
40 cycles of 1 min at 94°C, 35 s at 60°C, and 1 min at 72°C, with a final
elongation of 7 min at 72°C.

Identification of PCR products by RLB hybridization. Briefly, the collected amplicons of the 23S-5S rRNA spacer region of Rickettsia
were used in a reverse line blot hybridization assay in which specific amino-
oligonucleotide probes are covalently linked to activate a membrane in
parallel lines using a slotted miniblotter (MN 45). Hybridizations of the
denatured PCR product samples with the species-specific probes are de-
tected using chemiluminescence. The membrane was reused a maximum of
five times.

The preparation of the RLB membrane and the hybridization of oli-
gonucleotide probes (catch-all Rickettsia, SFG, R. conorii, Rickettsia slo-
vaca, Rickettsia aeschlimannii, Rickettsia rickettsii/sibrica, Rickettsia hel-
vetica, R. felis, TG, R. typhi, and Rickettsia prowazekii) containing a 5° C₂
amino linker extremity were carried out as previously described (7).

Positive controls were plasmas containing the 23S-5S intergenic
spatial sequence from R. prowazekii, R. conorii, and R. aeschlimannii,
kindly provided by Pedro Andra (Centro Nacional de Microbiologia, In-
stituto de Salud Carlos III, Spain), and cell culture DNA of R. rickettsii, R.
slowaca, R. felis, and R. typhi, kindly provided by Didier Raoult (Unité des
rickettsies, Faculté de médecine, Marseille, France). To monitor cross-
contamination and false-positive results, negative controls from DNA
dNA extraction and PCR amplification were included in each batch tested by
the PCR and RLB hybridization.

DNA sequencing and data analysis. To confirm the RLB results, 10
PCR products obtained from 7 ticks and 3 fleas, which were randomly
chosen and hybridized with species-specific probes, were sequenced with
targeting of the 23S-5S intergenic spacer using RLB-23S-5'-F/RLB-23S-5-
'R primers. In addition, DNA fragments obtained from the 7 ticks were
sequenced with targeting of the ompA gene using ompAF/ompAR primers
(10). The DNA of the 3 fleas was sequenced with targeting of the ompB
gene using MS9 and 807R primers (11). Furthermore, samples that hyb-
dridized only with the catch-all and SFG probes were sequenced using the
same primers.

The sequences from representative rickettsial species chosen to infer
the phylogenetic tree of the 23S-5S spacer were aligned using Clustal W
Mega 5.02 software. A phylogenetic tree was constructed by the maximum
parsimony method using MEGA version 5.02 software.

Statistical analysis. The chi-square test (EpiInfo 6.04) was used to
calculate the infection rate in R. sanguineus ticks, with 15% being the
predicted infection rate. The infection rate in R. sanguineus ticks was
significantly higher than the predicted rate at P < 0.05.

Nucleotide sequence accession numbers. Sequence data have been
deposited in GenBank; accession numbers for the partial 23S-5S inter-
genic spacer sequences and ompA gene are KF245433 to KF245444 and
KF245445 to KF245453, respectively.

RESULTS
Collection and identification of ticks and fleas. Eighty-six do-

comestic dogs, sheep, and goats from 8 municipalities of the Sfax
governorate were examined for the presence of hemophagous
potential arthropod vectors of Rickettsia. A total of 663 ticks and
470 fleas were removed from 38 infested animals (16 dogs, 12
sheep, and 10 goats) (Fig. 1). All 663 ticks were identified as Rhic-
picophilus sanguineus (159 males, 400 females, and 104 nymphs),
and all fleas were identified as Ctenocephalides felis (186 males and
284 females). R. sanguineus (n = 657) ticks were collected from 13
of the 28 dogs and from a single ovine (n = 6). C. felis fleas were
removed from 3 dogs (n = 40), 11 of the 14 sheep (n = 214), and
10 of the 40 goats (n = 216).

Detection of rickettsiae in collected arthropods by RLB. Four
hundred three of the 1,133 collected arthropods (198 R. san-
guineus ticks and 205 C. felis fleas) were analyzed for the presence
of Rickettsia DNA.

By RLB, the overall Rickettsia prevalence in the ticks and fleas
that were analyzed was 22.8% (92/403). A significant difference
was observed between the infection rate in R. sanguineus ticks,
37.4% (74/198), and that in C. felis fleas, 8.3% (17/205) (χ² = 36.2,
df = 1, P < 0.001) (Table 1).

R. sanguineus adults had a significantly higher rate of Rickettsia
infection (69/163, 42.3%) than did the nymphs (5/35, 14.3%) (χ² = 5,
P = 0.001) (Table 1). The Rickettsia prevalence in R.
sanguineus ticks varied among municipalities from 11.1% to
65.2%; the observed difference was statistically significant (χ² = 27.82,
P < 0.001). A total of 74 DNA samples, extracted from R.
sanguineus ticks, reacted with the Rickettsia catch-all and SFG
probes (Fig. 2), of which 69 (93.2%) hybridized with R. conorii
probes while the remaining 5 Rickettsia isolates, which did not
react with any specific probes, were sequenced.

R. conorii was detected in ticks collected on dogs and in all sites
where ticks were sampled (Table 1).

The 17 positive C. felis fleas were detected in five of eight sites
visited (Table 1). The samples with positive DNA showed hybrid-
ization with the catch-all and the specific SFG probes. RLB
allowed species identification of 15 flea samples: 13 hybridized with the
R. conorii probe and 2 with the R. felis probe. The two remaining
samples, which did not react with any of the specific probes,
were sequenced.

R. conorii was detected in C. felis fleas collected from sheep and
goats from 5 municipalities (Sakiet EzziT, Sfax Sud, Sfax Ouest,
Karkennah, and Malloulech) (Table 1). R. felis was identified in the fleas removed from a sheep and a goat in two sites (Sakiet EzziT
and Karkennah).

DNA sequencing and data analysis. Positive samples (5 ticks
and 2 fleas) showing no reaction with any of the specific probes
were analyzed by sequencing three fragments: the 23S-5S inter-
genic spacer, the ompA gene, and the ompB gene. Sequencing
allowed the identification of Rickettsia massiliae Bar 29 with 100% similarity to the partial 23S-5S sequence of the Spanish strain
(AY125014) in two ticks and no fleas. ompA gene sequencing for
these two infected R. sanguineus ticks confirmed the result and
also showed 100% similarity with R. massiliae Bar 29 (U43792).
These two ticks were collected from a sheep in Sakiet EzziT and
a dog in Jebeniana.

The sequencing of the 23S-5S spacer of randomly chosen R.
sanguineus ticks infected by R. conorii confirmed the infection of 5
of 7 ticks by the R. conorii Israeli spotted fever strain, which is
similar to the published shotgun sequence of R. conorii ISF strain
ISTT CDC1 contig09 (AJVP01000009.1). These 5 ticks were
collected from Jebeniana. These samples yielded positive PCR prod-
ucts for the ompA gene with 99% similarity with the Italian strain
Israeli tick typhus Rickettsia strain 3 (AY197564). The two remain-
ing ticks, collected from dogs in Karkennah and Malloulech, were
infected with R. conorii, and their sequences targeting the 23S-5S
spacar and the ompA gene showed 99% similarity with Rickettsia
conorii subsp. conorii (AE006914).

The sequenced PCR product of the 23S-5S spacer obtained from
three fleas confirmed the infection of two C. felis fleas
by *R. felis* with 100% similarity to the strain URRWFXCal2 (CP000053). These two fleas were collected from sheep in Sakiet Ezzit and Karkennah. The third sequence of a flea, collected from a goat from Sfax Ouest, was 100% identical to the partial sequence of the 23S-5S spacer of *R. conorii* subsp. *conorii* strain Malish 7 (AE006914). The *ompB* sequencing confirmed the species results obtained from 23S-5S sequencing for fleas infected by *R. felis*.

In a maximum parsimony analysis based on the alignment of the 23S-5S spacers of rickettsiae, a phylogenetic tree was constructed from sequences of the *Rickettsia* species and strains ob-

![FIG 1 Occurrence of arthropods and *Rickettsia* spp. in Sfax: Sfax region map showing the eight sampling areas and distribution of arthropods and *Rickettsia* species.](http://jcm.asm.org/)

<table>
<thead>
<tr>
<th>Municipality</th>
<th>Female (n/N)</th>
<th>Male (n/N)</th>
<th>Nymph (n/N)</th>
<th>Total n/N (%)</th>
<th>Rickettsia species identified (no.)</th>
<th>Female (n/N)</th>
<th>Male (n/N)</th>
<th>Total n/N (%)</th>
<th>Rickettsia species identified (no.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jebeniana</td>
<td>2/4</td>
<td>13/16</td>
<td>1/3</td>
<td>16/23 (65.2)</td>
<td><em>R. conorii</em> (15), SFG (1)</td>
<td>0/12</td>
<td>0/4</td>
<td>0/16 (0)</td>
<td></td>
</tr>
<tr>
<td>Hincha</td>
<td>3/5</td>
<td>10/17</td>
<td>1/1</td>
<td>14/23 (60.9)</td>
<td><em>R. conorii</em> (14)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>Malloulech</td>
<td>0/1</td>
<td>0/1</td>
<td>2/16</td>
<td>2/18 (11.1)</td>
<td><em>R. conorii</em> (1), SFG (1)</td>
<td>3/45</td>
<td>1/18</td>
<td>4/63 (6.3)</td>
<td>SFG (2), <em>R. conorii</em> (2)</td>
</tr>
<tr>
<td>Sfax Ouest</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td></td>
<td>1/4</td>
<td>0/4</td>
<td>1/8 (12.5)</td>
<td><em>R. conorii</em> (1)</td>
</tr>
<tr>
<td>Agareb</td>
<td>0/1</td>
<td>10/33</td>
<td>0/1</td>
<td>10/35 (28.6)</td>
<td><em>R. conorii</em> (10)</td>
<td>0/10</td>
<td>0/2</td>
<td>0/12 (0)</td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>14/52 (26.9)</td>
<td>55/111 (49.5)</td>
<td>5/35 (14.3)</td>
<td>74/198 (37.4)</td>
<td></td>
<td>12/122 (9.8)</td>
<td>9/83 (6)</td>
<td>17/205 (8.3)</td>
<td></td>
</tr>
</tbody>
</table>

*Symbols and abbreviations: —, no samples; n, number of positive DNA samples; N, number of tested specimens; (%), % prevalence.*
tained from GenBank. Phylogenetic studies currently show that several groups can be determined within the genus Rickettsia. Figure 3 shows that sequences from Tunisian samples were clustered in three groups. Six sequences isolated from ticks (KF245435 to KF245440 and KF245442) and one flea (KF245443) were clustered in the first group, which contains most of the SFG Rickettsiae species (R. conorii subsp. conorii, Rickettsia conorii ISF strain, Rickettsia sibirica strain Mongolotimonae, Rickettsia africana, Rickettsia parkeri, R. slovaca, R. rickettsii, Rickettsia honei, and Rickettsia japonica). Two other sequences (KF245434 and KF245444), isolated from two ticks infected by R. massiliae Bar 29, were included in the second group (R. massiliae Bar 29, Rickettsia rhipicephali, R. aeschlimannii, and Rickettsia montanensis). Finally, sequences from two fleas infected by R. felis (KF245433 and KF245441) were clustered in the third group, which contains R. felis, Rickettsia australis, and Rickettsia akari.

**DISCUSSION**

In this study, using molecular tools (RLB and sequencing), we report evidence of spotted fever group rickettsiae among ectoparasites collected from domestic animals in the Sfax governorate.

Tick and flea species removed from dogs and small ruminants were identified as Rhipicephalus sanguineus and Ctenocephalides felis. The occurrence of these two arthropods in this region could be attributed to the presence of favorable climatic conditions and adaptation to hosts (dogs, sheep, and goats) (12, 13). *R. sanguineus*, a three-host tick, is mainly collected from dogs, its preferred host (13), and only a few specimens were found on small ruminants. This tick is well adapted to human rural and urban environments because of its association with dogs, considered to be the main reservoir of *R. conorii* (14). In contrast, *C. felis*, a ubiquitous ectoparasite, was found on all the prospected animals (dogs, sheep, and goats).

To detect *Rickettsia*, several PCRs targeting a variety of genes were described. However, the intergenic spacer 23S-5S rRNA showed higher sensitivity since it has a high copy number in the genome, making it easy to amplify from even small quantities of DNA. Moreover, it is widely used in taxonomy and suitable for the clarification of phylogenetic relationships within the SFG even between closely related species (15).

RLB or PCR-reverse blot hybridization assay was described as a very sensitive and specific test (7) and a fast, reliable technique (16) for the simultaneous detection and identification of pathogens. Using this method, we confirmed that *Rickettsia* infection is prevalent in *R. sanguineus* ticks and *C. felis* fleas collected in the Sfax area. *R. sanguineus* had a significantly higher rate of *Rickettsia* infection than did fleas. This species of tick is considered to be the main vector and reservoir of the *R. conorii* complex in the Mediterranean region: Spain, Italy, Greece, France, and Portugal (17, 18).

The prevalence of *Rickettsia* infection in *R. sanguineus* (37.4%) is roughly similar to that reported in central Spain (25%) (19) but higher than that recorded in North African countries such as Morocco (4.7%) (20) and in some European countries, such as Greece (2.4%) and Cyprus (8%) (21, 22). This large variation among the reported prevalence of *Rickettsia* could be related to the differences in methodologies used, the biotope of the vectors, and the abundance of *Rickettsia* reservoirs. Differences in the prevalence of *Rickettsia* in ticks were observed among the municipalities, with the highest prevalence detected in Karkennah (83.3%), followed by Jebeniana (65.2%). For Karkennah, the results should be confirmed by other sampling since all ticks were collected from the same dog. For Jebeniana, the observed rate may be related to geographic and socio-occupational factors, especially the fact that family incomes were based on livestock. The infection rate of adult ticks was statistically higher than that of nymphs, a fact that was also demonstrated by a xenodiagnostic study (14).

Since the first description of *R. conorii* (in *R. sanguineus* ticks) by Brumpt in 1932 in Tunisia (23), no recorded report has confirmed the presence of this bacterium in ticks. However, *R. conorii* was detected by molecular tools in patients in Tunisia (6, 24). In our study, species-specific probes showed that 34% of DNA samples from *R. sanguineus* ticks, collected from dogs in the Sfax governorate, contained DNA corresponding to *R. conorii*. In Algeria, a neighboring country, Bitam et al. reported an infection rate of 26% for this bacterium in *R. sanguineus* ticks (25), which is higher than those reported in northern Mediterranean regions such as Bulgaria, Turkey, and Albania (14% (26).

In the Mediterranean region, dogs show a high prevalence of infection with *R. sanguineus* ticks and the close proximity of animals and humans is a risk factor for the *Rickettsia* infection (1). Znaen et al. (27) have reported 37 cases of SFG rickettsiae in the Sfax region based on clinical and serological data. In 19 of these cases, there was reported contact with animals (27). In Morocco, among 45 patients, 34 reported contact with dogs and 29 had *R. conorii* subsp. *conorii* antibodies or a positive skin biopsy specimen (28). In Algeria, Mouffok et al. (29) reported that 161 rick-
ettiosis cases had been exposed to dogs. Of these, 58% of the patients had an *R. conorii* infection (29). Moreover, in our study, 2 of 6 *R. sanguineus* ticks collected on sheep in Sakiet Ezzit were infected by *R. conorii*. In Cyprus, Chochlakis et al. (22) also found that the prevalence of infection in *R. sanguineus* ticks collected from sheep and goats was significantly higher than in those from dogs. *R. conorii* has also been detected in several other countries in other tick species such as *Rhipicephalus bursa*, *Dermacentor marginatus*, and *Ixodes ricinus* (17).

In addition to *R. conorii* subsp. *conorii*, *R. sanguineus* ticks were also infected by the *Rickettsia* Israeli spotted fever strain (AY197564). This *Rickettsia* strain was first isolated from ticks in Palestine in 1946 (30). This strain has been classified with *R. conorii* subsp. *conorii*, *Rickettsia conorii* subsp. *indica*, and *Rickettsia conorii* subsp. *caspias* as a new subspecies within *R. conorii* on the basis of multilocus sequence typing (31). Recently, in Israel, Har- 

![Phylogenetic tree based on the studies of 23S-5S intergenic spacer of bacteria of the genus Rickettsia using the MEGA 5.02 software. The tree was obtained using the maximum parsimony method. The numbers at the nodes are the proportions of 100 bootstrap resamplings that support the topology shown. The TUN sequences detected in this work have been deposited in GenBank under accession numbers KF245433 and KF245444, respectively. The sequences used for comparison were obtained from GenBank. TUN, Tunisia; ○, tick samples; ●, flea samples.](http://jcm.asm.org/)

FIG 3
thought since it occurs in Tunisia. Indeed, in a recent study, Zna zen et al. reported 2 cases of ISF from patients with fever, headache, and arthromyalgia confirmed by the detection of rickettsial DNA in skin biopsy samples (24).

In this study, a third Rickettsia species, R. massiliae strain Bar 29, was detected in three R. sanguineus ticks, collected from sheep and dogs from Sakiet Ezzit and Jebeniana. This Rickettsia strain was first isolated in 1996 from R. sanguineus in Barcelona, Spain (18). Moreover, a strain close to R. massiliae was isolated from R. sanguineus ticks collected in Arizona (United States) (34). This strain, which is close to R. massiliae, is identical to an isolate previously described as MTU5 and slightly different from R. massiliae by antigenic and phenotypic criteria (35). Indeed, this is the first report of R. massiliae Bar 29 isolated from R. sanguineus in North Africa. However, R. massiliae was detected in R. sanguineus in Morocco with a prevalence of 4.7% (20) and in Algeria (25).

In addition to R. conorii, we also detected R. felis in two specimens of C. felis. R. felis has been associated with fleas worldwide, in Algeria and Morocco (37), Italy (38), and the United States (39). However, in this investigation, the prevalence of R. felis (1%) is lower than that reported in Morocco (20%) in C. felis fleas removed from sheep, cats, and dogs [37] and in Italy (11.9% in dog and cat fleas [38]).

The presence of R. felis in fleas collected from domestic animals in the Sfax region, ticks could transmit MSF disease, as could C. felis fleas, which may play an important role. To our knowledge, this is the first report that shows the circulation of the etiological agent of MSF in C. felis fleas in the world.

In addition to R. conorii, we also detected R. felis in two specimens of C. felis. R. felis has been associated with fleas worldwide, in Algeria and Morocco (37), Italy (38), and the United States (39). However, in this investigation, the prevalence of R. felis (1%) is lower than that reported in Morocco (20%) in C. felis fleas removed from sheep, cats, and dogs [37] and in Italy (11.9% in dog and cat fleas [38]).

This study provides current data about the rickettsial species and their arthropod vectors that can represent a risk for humans and animals in Tunisia. This is the first report describing the presence of the R. conorii ISF strain and R. massiliae strain Bar 29 in R. sanguineus ticks and R. conorii and R. felis in C. felis, collected in Tunisia. With respect to public health, R. sanguineus and C. felis, both of which are abundantly present, seem to represent the most significant tick and flea species carrying Rickettsia agents and may play an important role in maintaining rickettsial infections. Further investigations in humans and animals are needed and must be compared to these data.

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