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ORIGINAL ARTICLE

First molecular evidence of *Borrelia burgdorferi* sensu lato in goats, sheep, cattle and camels in Tunisia

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Abstract

Borrelia burgdorferi sensu lato (s.l.) are tick-transmitted spirochaetes of veterinary and human importance. Molecular epidemiology data on ruminants are still lacking in most countries of the world. Therefore, the aim of this study was to estimate the rate of *B. burgdorferi* s.l. infection in ruminants from Tunisia. A total of 1,021 ruminants (303 goats, 260 sheep, 232 cattle and 226 camels) from different bioclimatic areas in Tunisia were investigated for the presence of *B. burgdorferi* s.l. DNA in blood by real time PCR. Prevalence rates were 30.4% (92/303) in goats, 6.2% (16/260) in sheep, 1.3% (3/232) in cattle, and 1.8% (4/226) in camels. Only tick species belonging to *Rhipicephalus* and *Hyalomma* genera were found on the investigated animals. In small ruminants, the prevalence of *B. burgdorferi* s.l. varied significantly according to localities and farms. Goats located in humid areas were statistically more infected than those located in sub-humid areas. Prevalence rates varied significantly according to age and breed in sheep, and age and tick infestation in goats. This study provides the first insight into the presence of *B. burgdorferi* s.l. DNA in ruminants in Tunisia, and demonstrates that host species such as goats and sheep may play an important role in natural Lyme disease cycles in this country.

Key words

Borrelia burgdorferi sensu lato, ruminant species, molecular evidence, risk factors, Tunisia

INTRODUCTION

Lyme borreliosis, a zoonotic tick-borne disease caused by spirochaetes of the group *B. burgdorferi* sensu lato, is a multi-organ disease of mammals widespread in the northern hemisphere [1]. The clinical form of Lyme borreliosis occurs in humans and domestic animals, especially dogs, horses, and cattle [2, 3, 4]. *Borrelia burgdorferi* s.l. includes at least 19 genospecies, with 5 of them known to be pathogenic for humans: *Borrelia burgdorferi* sensu stricto (s.s.), *Borrelia afzelii*, *Borrelia bavariensis*, *Borrelia garinii* and *Borrelia spielmanii*[5, 6, 7, 8].

Most reports on Lyme borreliosis in domestic animals focus on dogs and horses [3, 4] and only few descriptions of infection in cattle and sheep are available [9, 10]. In cattle, lyme borreliosis was reported in the 1980s in the USA [2, 11]. The predominant clinical signs of infection included lameness, weight loss and abortion. A few cases of *B. burgdorferi* s.l. infection have been reported in sheep, with lameness, anorexia, and poor body condition [10].

Although most ruminants are considered as incompetent reservoirs [12], they have some importance in the ecology and dynamics of the circulation of *B. burgdorferi* s.l., especially by acting as maintenance hosts for tick populations, as spreaders of infected ticks [13], or as a potential host of ticks that could infect themselves by co-feeding, as observed in sheep [14, 15].

This complex has been weakly investigated in Tunisia, and its current geographic distribution remains unknown. *Ixodes ricinus* ticks, the main vector of *B. burgdorferi* s.l. in Europe, are abundant in the humid regions of Tunisia [16], and are very frequently infected by *Borrelia lusitaniae* [17, 18], a species which although considered non-pathogenic has been isolated from a patient in Portugal [19].

Several direct and indirect methods are used for detecting the *B. burgdorferi* s.l. complex. Indirect methods, mainly serological, are based on antibody detection [20]. Direct methods detect the bacteria or its components. The methods most often used are culture, microscopy and molecular; indeed, DNA detection by PCR has been acclaimed for its high sensitivity and specificity [21, 22].

OBJECTIVE

To the best of our knowledge, no information is available on the presence of *B. burgdorferi* s.l. complex in ruminants in Tunisia. Therefore, this study aims to understand the role of ruminants as carriers and potential spreaders of Lyme disease in this country. The exposure of several ruminants' species to *B. burgdorferi* s.l. complex was investigated using Real time PCR while also taking into account geographic and host-relating factors.

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MATERIALS AND METHOD

Ruminant populations and study regions

Sheep. In May 2011, blood samples were collected from 260 randomly selected sheep which belonged to 9 herds situated in 2 localities of the governorate of Bizerte, a sub-humid bioclimatic area with a mean annual rainfall of 400 mm: El Alia (37°16′ N, 10°03′ E) and Khetmine (latitude 37°16′ N, longitude 9°99′ E) (Fig. 1). The sheep belonged to 6 breeds: Barbarine (118), Noire de Thibar (82), Queue Fine de l'Ouest (10), Merinos (2), Sicilo-sarde (1) and cross-breeds (47). Gender ratio (male/female) was 0.24 and mean age 4.9±2.0 years.

Goats. Blood sampling was performed from May to September 2013 on 303 apparently healthy goats from 16 herds situated in 4 localities of the Bizerte and Beja governorates: El Alia (Governorate of Bizerte, latitude 37°16′ N, longitude 10°03′ E), belonging to the sub-humid bioclimatic area, Joumine (Governorate of Bizerte, latitude 36°92′ N, longitude 9°38′ E), Sejnane (Governorate of Bizerte, latitude 37°15′ N, longitude 9°23′ E), and Amdoun (Governorate of Beja, latitude 36°76′ N, longitude 9°08′ E). The 3 latter localities belong to the humid bioclimatic area with a mean annual rainfall of 650 mm (Fig. 1). Goats breeds were: a local breed (n=275), Alpine (n=23) and Maltese (n=5). Goats' gender ratio (male/female) was 0.3 and mean age 3.9±1.7 years.

Cattle. From July to December 2012, blood was collected from 232 randomly selected cattle in 36 farms situated in three localities of the Bizerte governorate in northern Tunisia: Utique (latitude 37°16′ N, longitude 9°52′ E) belonging to the semi-arid bioclimatic area with a mean annual rainfall of 400 mm, El Alia (latitude 37°16′ N, longitude 10°03′ E) and Mateur (latitude 37°02′ N, longitude 9°39′ E) belonging both to the sub-humid bioclimatic area with a mean annual rainfall of 600 mm (Fig. 1).

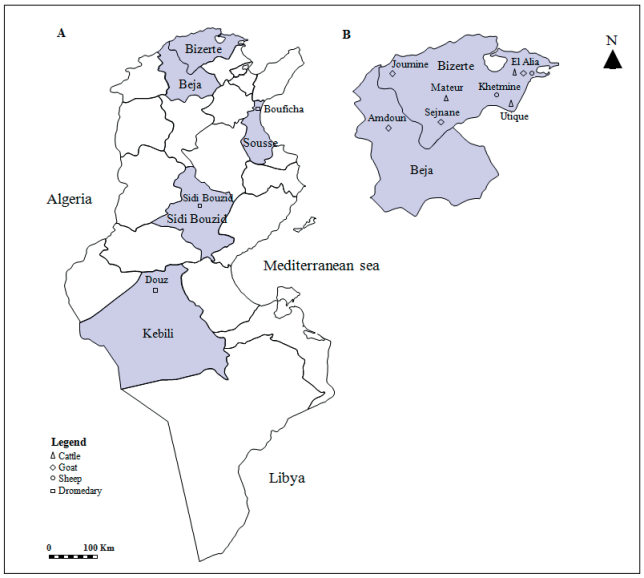

Figure 1. Map of Tunisian studied localities
A. Map of Tunisia showing location of the governorates of Bizerte, Beja and the localities of Boufícha, Sidi Bouzid and Douz located in the governorates of Sousse, Sidi Bouzid and Kebili, respectively. B. Map of Bizerte and Beja governorates showing the location of El Alia, Khetmine, Utique, Mateur, Joumine, Sejnane and Amdoun localities

Camels. From May to October 2009, blood sampling was performed on 226 apparently healthy camels situated in 3 localities: Boufícha (Governorate of Sousse, latitude 36°18′ N, longitude 10°27′ E) belonging to the semi-arid bioclimatic area with a mean annual rainfall of 350 mm, Sidi Bouzid (Governorate of Sidi Bouzid, latitude 35°0′ N, longitude 9°29′ E) belonging to the arid bioclimatic area with a mean annual rainfall of 237 mm, and Douz (Governorate of Kebili, latitude 33°27′ N, longitude 9°01′ E) belonging to the Saharan bioclimatic area with a mean annual rainfall of 89 mm (Fig. 1).

Blood sampling, tick collection and DNA extraction. Blood samples were collected from the jugular vein into EDTA tubes (Becton Dickinson). For each ruminant, the studied region, approximate age, gender, breed and presence or absence of ticks, were noted. Ticks were collected from all animals, preserved in 70% ethanol and identified to genus and species levels by using the taxonomic keys of Walker et al. [23]. DNA was extracted from 300 µl of EDTA-preserved whole blood using the Wizard® Genomic DNA purification kit (Promega, Madison, USA), according to the manufacturer's instructions. DNA yields were determined with a spectrophotometer (Jenway, Genoa, Italy). Isolated DNA was stored at -20 °C until use.

Real time PCR detection of *Borrelia burgdorferi* sensu lato. All ruminants were screened for the presence of *B. burgdorferi* s.l. by using complex-specific primers and a TaqMan probe, as described by Courtney et al.[22]. A 75-bp fragment was amplified in the *B. burgdorferi* s.l. 23S rRNA gene. Real time PCR was performed using Premix Ex Taq ™ (Perfect Real Time) (Takara, Mirus Bio, Madison, WI, USA) in a 7500/7500 Fast Real-Time PCR System quantitative thermal cycler (Applied Biosystems, USA). PCR amplification was performed in a simplex format by optimal reaction conditions using primers Bb23Sf and Bb23Sr at 700 nM each, probe Bb23Sp-FAM at 175 nM, and 2 µl of template DNA. For all reactions, cycling conditions included an initial activation of the *Taq* DNA polymerase at 95 °C for 15 min, followed by 45 cycles of 1 min denaturation at 95 °C, followed by a 1 min annealing-extension step at 60 °C. Negative and positive controls were included in all runs.

Statistical analyses. Exact confidence intervals (CI) for prevalence rates at the 95% level were calculated. To study the difference of the molecular prevalence of *B. burgdorferi* s.l. according to bioclimatic zone, locality, farm, gender, age, breed and presence or not of ticks, chi square test or Fisher's exact test were performed using Epi Info 6.01 (CDC, Atlanta, USA), with a cutoff value of 0.05. In order to consider any confusion factor, a chi square Mantel-Haenszel test was performed.

RESULTS

Ticks' collection. In this cross-sectional study, a total of 241 (92.7%) sheep and 113 (37.3%) goats were infested by at least one tick species. Ticks infecting sheep belonged to 3 species of *Rhipicephalus*: *R. turanicus*, *R. sanguineus*, and *R. annulatus*. Ticks collected from goats belonged to 2 genera and 4 species, namely, *R. turanicus*, *R. bursa*, *R. sanguineus* and *Hyalomma*

Table 1. Prevalence of *Borrelia burgdorferi* sensu lato according to governorates, localities and farms in goats and sheep in Tunisia

Host	Governorate	Locality	Farm No.	No. of animals	Positive (%±C.I.[1])
Goats	Bizerte	Alia	1	30	0 (0)
			2	09	0 (0)
			3	15	0 (0)
			4	16	1 (6.3±0.12)
			Total	70	1 (1.4±0.03)
		Sejnane	9	23	1 (4.3±0.08)
			10	25	15 (60.0±0.19)
			11	32	17 (53.1±0.17)
			Total	80	33 (41.3±0.11)
		Joumine	12	19	11 (57.9±0.22)
			13	07	5 (71.4±0.33)
			14	20	8 (40.0±0.22)
			15	20	14 (70.0±0.20)
			16	15	4 (26.7±0.22)
			Total	81	42 (51.9±0.11)
		Total		**232**	**76 (32.8±0.06)**
	Beja	Amdoun	5	16	0 (0)
			6	24	0 (0)
			7	15	9 (60±0.25)
			8	17	7 (41.2±0.23)
		Total		**72**	**16 (22.2±0.10)**
	Total			**303**	**92 (30.4±0.05)**
Sheep	Bizerte	Alia	1	30	0 (0)
			2	20	0 (0)
			3	30	1 (3,3±0.06)
			8	30	0 (0)
			9	30	3 (10±0.11)
			Total	140	4 (2.6±0.03)
		Khetmine	4	30	0 (0)
			5	30	8 (26.7±0.16)
			6	30	4 (13.3±0.12)
			7	30	0 (0)
			Total	120	12 (10±0.05)
		Total		**260**	**16 (6.15±0.03)**

[1] C.I.: 95% confidence interval.

excavatum. Ticks collected from cattle were classified as *Hyalomma marginatum*, *H. excavatum* and *H. scupense*. *Rhipicephalus* spp. engorged females were collected, but were not identified to species. Only 21 of the 232 (9.1%) examined cattle were infested by at least one of these tick species. Ticks were observed in 85 camels (37.6%) and identified as *H. dromedarii*, *H. excavatum* and *H. impeltatum*.

Molecular survey of *Borrelia burgdorferi* sensu lato. In goats, the *B. burgdorferi* s.l. overall infection rate was 30.4% (92/303) (Tab. 1). A statistically significant difference was noted among bioclimatic areas (p<0.001), localities (p<0.001) and farms (p<0.001). Goats located in humid areas (39.1%; 91/233) represented by the localities of Sejnane, Joumine and Amdoun were statistically more infected than those located in a sub-humid area (1.4%; 1/70), represented by the locality of El Alia. The highest prevalence was observed in Joumine

Table 2. Factors associated with molecular prevalence of *Borrelia burgdorferi* sensu lato in goats and sheep

	Goats			Sheep		
Risk factor	No.	Positive (%±C.I.[1])	P-value	No.	Positive (%±C.I.[1])	P-value
Gender			0.267			0.207
Male	70	25 (35.7±0.11)		50	5 (10.0±0.08)	
Female	233	67 (28.8±0.06)		210	11 (5.2±0.03)	
Age			0.000*			0.002*
≤ 2 years	133	56 (42.1±0.08)		63	9 (14.3±0.09)	
> 2 years	170	36 (21.2±0.06)		197	7 (3.5±0.02)	
Breed			0.052			0.002*
Local/Barbarine[2]	275	88 (32.0±0.06)		118	13 (11.0±0.06)	
Other breeds[3]	28	04 (14.3±0.13)		142	3 (2.1±0.02)	
Tick infestation			0.000*			0.275
Infested	113	16 (14.2±0.06)		244	14 (5.7±0.03)	
Not infested	190	76 (40.0±0.07)		16	2 (12.5±0.16)	
Total	**303**	**92 (30.4±0.05)**		**260**	**16 (6.2±0.03)**	

[1] C.I.: 95% confidence interval.
[2] Local for goats and Barbarine for sheep.
[3] Other breeds are Alpine and Maltese for goats and Noire de Thibar, Queue fine de l'Ouest, Merinos, Sicilo-sarde and crossbred for sheep.
* Significant test.

(51.9%, 42/81) (governorate of Bizerte), and farm No. 13 (Joumine locality) was the most infected farm (71.4%, 5/7) (Tab. 1). Analysis of risk factors showed that *B. burgdorferi*s.l. prevalence was higher in young goats (≤2 years) (42.1%; 56/133) than in adults (21.2%; 36/170) (p<0.001) (Tab. 2). Goats infested by ticks were statistically less infected by *B. burgdorferi* s.l. (14.2%; 16/113), compared to those free of ticks (40%; 76/190) (p<0.001) (Tab. 2).

In sheep, the *B. burgdorferi*s.l. overall infection rate was 6.2% (16/260). A statistically significant difference was noted among localities (p=0.016) and among farms (p<0.001). The highest prevalence was observed in Khetmine (governorate of Bizerte) (10%; 12/120), and farm No. 5 (Khetmine locality) was the most infected farm (26.7%; 8/30) (Tab. 1). *B. burgdorferi* s.l. prevalence was higher in young sheep (≤2 years) (14.3%; 9/63) than adults (>2 years) (3.5%; 7/197) (p=0.002) (Tab. 2). Additionally, the Barbarine breed (11.0%; 13/118) was the most frequently infected, compared to other breeds (2.1%; 3/142) (p=0.002) (Tab. 2). As for other ruminants, *B. burgdorferi* s.l. was detected in 4 adult camels (1.8%; 4/232) (2 females from Bouficha, belonging to a semi-arid area, and 2 males from Douz belonging to the arid area), and 3 adult cattle (1.3%; 3/226) (2 females from Mateur and 1 female from El Alia both, belonging to the sub-humid area) (Tab. 3, 4).

DISCUSSION

To-date, there is still a lack of comprehensive knowledge on the distribution and prevalence of *B. burgdorferi* s.l. among ruminants in North Africa; therefore, in an attempt to fill this gap, the presented study reports for the first time the presence of *B. burgdorferi* s.l. in sheep, goats, cattle and camels in Tunisia. The results show that in sheep the prevalence of *B. burgdorferi* s.l. in El Alia and Khetmine were 2.6 and

Table 3. Prevalence of *Borrelia burgdorferi* sensu lato according to bioclimatic zones, governorates and localities in cattle and camels in Tunisia

Host	Bioclimatic zone	Governorate	Locality	No. of animals	Positive (%±C.I.[1])
Cattle	Sub-humid	Bizerte	Alia	70	1 (1.4±0.03)
			Mateur	76	2 (2.6±0.03)
			Total	146	3 (2.0±0.02)
	Semi-arid		Utique	86	0 (0)
	Total			**232**	**3 (1.3±0.01)**
Camels	Semi-arid	Sousse	Bouficha	32	2 (6.2±0.08)
	Arid	Sidi Bouzid	Sidi Bouzid	155	0 (0)
	Saharan	Kebili	Douz	39	2 (5.1±0.07)
	Total			**226**	**4 (1.8±0.02)**

[1] C.I.: 95% confidence interval.

Table 4. Factors associated with molecular prevalence of *Borrelia burgdorferi* sensu lato in cattle and camels

	Cattle			Camels		
Risk factor	*No.*	*Positive (%±C.I.[1])*	*P-value*	*No.*	*Positive (%±C.I.[1])*	*P-value*
Gender			0.629			0.641
Male	33	0 (0)		120	2 (1.7±0.02)	
Female	199	3 (1.5±0.02)		106	2 (1.9±0.02)	
Age			0.477			0.112
< 1 / 2[2] years	30	0 (0)		44	0 (0)	
[1 / 2[2]–7] years	156	3 (1.9±0.02)		109	4 (3.7±0.03)	
> 7 years	46	0 (0)		73	0 (0)	
Tick infestation			0.249			0.153
Infested	21	1 (4.8±0.09)		84	0 (0)	
Not infested	211	2 (0.9±0.01)		142	4 (2.8±0.03)	
Total	**232**	**3 (1.3±0.01)**		**226**	**4 (1.8±0.02)**	

[1] C.I.: 95% confidence interval.
[2] 1 year for cattle; 2 years for camels.

10.0%, respectively, with an average prevalence of 6.2%. This value is higher than the molecular prevalence reported by Chu et al. [24] in Chinese sheep kids (3.6%), and lower than that found by Fu et al. [25] in sheep from China (39.0%). However, most previous studies have been conducted using the serology method. The seroprevalence of *B. burgdorferi* s.l. was 23.8% among sheep in Egypt [26], 22.1% in Turkey [27], 16.7% in Slovakia [28], 14.1% in Italy [29], and 15.8–22.5% in China, as respectively reported by Li et al.[30] and Hua et al. [31]. Although sheep are considered an incompetent reservoir and do not seem to be able to transmit *B. burgdorferi* s.l. to ticks [28, 29], this animal species may represent a threat to humans by acting as a host maintaining tick populations, and as a spreader transferring pathogens from infected to uninfected ticks co-feeding on the same animal [15].

The *B. burgdorferi* s.l. molecular survey in goats showed that the prevalence in El Alia, Amdoun, Sejnane and Joumine were 1.4, 22.2, 41.3 and 51.9%, respectively, with an average of 30.4%. To the best of our knowledge, this is the first molecular detection of *B. burgdorferi* s.l. in goats. To-date, the role of goats as competent reservoirs remains obscure. The high *B. burgdorferi* s.l. prevalence in goats indicates that goats were exposed to *B. burgdorferi* s.l. infection and that they might act as reservoir hosts for borreliosis. Using serological methods, several reports showed the detection of *B. burgdorferi* s.l. antibodies in goats. Seroprevalence has been estimated at 36.8% in Italy [29], 18.4% in Slovakia [28], 18.0% in Egypt [26], 5% in Bolivia [32], and 19.1, 20.3 and 22.2% in China, as reported by Long *et al.* [33], Li et al. [30] and Zhang et al. [34], respectively.

In the current study, a significant difference in *B. burgdorferi* s.l. infection rates in goats was recorded among bioclimatic areas. Goats located in humid areas, represented by the localities of Sejnane, Joumine and Amdoun, were statistically more infected than those located in a sub-humid area, represented by the locality of El Alia. This difference, which has been reported in other studies on horses [35] and pigs [36], is probably related to the effect of bioclimatic conditions on the distribution of tick vectors [37]. This study shows that goats infested by *Rhipicephalus* and *Hyalomma* ticks were statistically less infected by *B. burgdorferi* s.l. than those free of ticks. This result suggests that these tick species may not be involved or only weakly involved in the transmission of this complex, and that other tick species and/or blood-feeding insects, such as bot-flies, fleas, and mosquitoes [38], are probably more incriminated in the transmission of *B.burgdorferi* s.l. in these investigated regions. Indeed, in Tunisia, *Ixodes ricinus* ticks are considered as the natural vector of *B. burgdorferi* s.l., especially *B. lusitaniae* [17, 39, 40, 41, 42]. Although ticks of this genus are known to be present in the Tunisian humid areas, none were found during this survey, probably because their peak activity occurs in autumn (November-October) [16]. This was previously observed by Fridriksdottir et al. [9], who reported the presence of seropositive animals in areas where *I. ricinus* ticks had never been recorded previously. In addition, overall prevalence rates of *B. burgdorferi* s.l. differed statistically according to localities and among sheep and goat farms. This discrepancy may result from differences in ecological factors, tick control programs, habitat types, husbandry practices, wildlife reservoir hosts, and/or microclimate, which may locally determine tick and host abundance [43, 44, 45].

Prevalence of *B. burgdorferi* s.l. infection was significantly affected by the age of the sheep and goats. Young animals were significantly more infected than adults, suggesting that the analyzed small ruminants were exposed to the spirochetes early in life, and that borreliacidal activity of the animal complement and/or antibodies probably increased according to age. This result is in agreement with a serological study on Norwich sheep which reported that the majority of studied animals appeared to become infected during the first 2 years of life [9]. In sheep, the Barbarine breed is the most infected by *B. burgdorferi* complex, probably because sheep of this breed are not in their natural environment, which is the steppe of dry land in Central Tunisia, belonging mainly to the pre-Saharan area [46].

B. burgdorferi s.l. was detected in 3 adult cattle (1.3%) situated in a sub-humid area. To our knowledge, this is the first report demonstrating the presence of *B. burgdorferi* s.l. DNA in cattle in Africa. Using the serological method, *B. burgdorferi* s.l. has also been suspected in other countries, such as Brazil (54.9%) [47], Germany (33%) [48], Slovakia (25.2%) [49], Japan (20%) [50] and the USA (7 and 71%) [51, 52]. Positive serological or molecular tests provide support to diagnosis, but are not conclusive of the presence of current infection or clinical disease [20]. In fact, Lyme borreliosis in cattle is probably an infrequent disease that is difficult

to diagnose due to the onset of unspecific symptoms [53]. In addition, cattle are not considered as reservoir hosts of *B. burgdorferi* s.l. [54], and the detection of some positive cases is probably due to the presence of other hosts in the same location [55].

B. burgdorferi s.l. DNA was detected in 1.8% of camels. This is the first report concerning *B. burgdorferi* s.l. in camels. Camels were exposed to *B. burgdorferi* s.l. infection and may be part of the natural cycle of this species complex in arid and Saharan areas. The absence of *I. ricinus,* which is one of the most incriminated tick species in the transmission of *B. burgdorferi* s.l. in Tunisia [17, 44], suggests that other vectors could transmit Lyme borreliosis in the ecosystems of camels.

CONCLUSION

This study demonstrates that *B. burgdorferi* s.l. DNA can be commonly found in Tunisian ruminants. Further studies are needed to determine the role of these animal species in the natural transmission of this complex to definitive hosts in Tunisia, and to identify and characterize the different genospecies infecting each ruminant species in the country.

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