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Prevalence and Host Specificity of Haemosporidian Parasites in Eurasian Kestrels and Eurasian Buzzards in Northern Italy: Morphological and Molecular Analysis

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ABSTRACT.—We investigated the prevalence and host specificity of haemosporidian parasites in wild Eurasian Kestrels (*Falco tinnunculus*) and Eurasian Buzzards (*Buteo buteo*) admitted to a recovery facility in northern Italy. By combining morphological and molecular approaches, we analyzed 47 blood samples (37 kestrels and 10 buzzards) in 2021–2023. We documented a notable prevalence of haemosporidian parasites, specifically *Haemoproteus* and *Leucocytozoon*. Of the 47 samples analyzed, 22 were positive for haemosporidian parasites, based on Giemsa-stained smears. The molecular procedure identified an additional four samples as positive, all of which had been previously classified as negative using the Giemsa-stained method, and allowed the analysis of three samples that had been excluded due to poor quality. In total, 27 birds (57%) tested positive for at least one haemosporidian: *Haemoproteus brachiatus* (22 samples, primarily from kestrels) or *Leucocytozoon* spp. (8 samples, all from buzzards). Three buzzards were infected with both genera. *Haemoproteus brachiatus* was detected in 48.6% of kestrels and 40% of buzzards. This study highlights the importance of combining morphological and molecular techniques to gain a comprehensive understanding of parasite prevalence among raptors admitted to a rehabilitation facility.

KEY WORDS: *cytochrome B*; *raptors*; *recovery facility*; *rehabilitation*.

PREVALENCIA Y ESPECIFICIDAD DE HOSPEDADOR DE PARÁSITOS HEMOSPÓRIDIOS EN *FALCO TINNUNCULUS* Y *BUTEO BUTEO* EN EL NORTE DE ITALIA: ANÁLISIS MORFOLÓGICO Y MOLECULAR

RESUMEN.—Investigamos la prevalencia y la especificidad de hospedador de parásitos hemospóridios en individuos silvestres de *Falco tinnunculus* y *Buteo buteo* ingresados en un centro de rehabilitacion ubicado en el norte de Italia. Combinando enfoques morfológicos y moleculares, analizamos 47 muestras de sangre (37 de *F. tinnunculus* y 10 de *B. buteo*) entre 2021 y 2023. Documentamos una prevalencia notable de parásitos hemospóridios, específicamente de *Haemoproteus* y *Leucocytozoon*. De las 47 muestras analizadas, 22 resultaron positivas a parásitos hemospóridios según las extensiones teñidas con Giemsa. El procedimiento molecular identificó como positivas a otras cuatro muestras, todas ellas previamente clasificadas como negativas mediante el método de Giemsa, y permitió el análisis de tres muestras adicionales que habían sido excluidas por mala calidad. En total, 27 aves (57%) resultaron positivas a por lo menos un hemospóridio: *Haemoproteus brachiatus* (22 muestras, principalmente de *F. tinnunculus*) o

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Leucocytozoon spp. (8 muestras, todas de *B. buteo*). Tres individuos de *B. buteo* estaban coinfectados con ambos géneros. *Haemoproteus brachiatus* fue detectado en el 48,6% de los individuos de *F. tinnunculus* y en el 40% de los individuos de *B. buteo*. Este estudio resalta la importancia de combinar técnicas morfológicas y moleculares para obtener una comprensión completa de la prevalencia de parásitos en rapaces ingresadas en un centro de rehabilitación.

[Traducción de los autores editada]

INTRODUCTION

Haemosporidian parasites, including *Haemoproteus* spp., *Plasmodium* spp., and *Leucocytozoon* spp., are a diverse and widespread group of haematic parasites transmitted by vectors such as louse flies (Hippoboscidae), biting midges (Ceratopogonidae), black flies (Simulidae), and mosquitoes (Culicidae) (Valkiūnas 2004, Yabsley et al. 2018). Over 200 haemosporidian species have been identified in avian hosts. *Leucocytozoon*, *Plasmodium*, and *Haemoproteus* are the most prevalent and widely distributed genera (Martinsen et al. 2006, Braga et al. 2011, Santiago-Alarcon et al. 2012). Despite their broad distribution, the impact of these parasites on wild raptors, both diurnal and nocturnal, remains under-studied (Cruz et al. 2024).

These parasites can significantly impact bird health, potentially causing symptoms such as weight loss, anemia, and even death, especially in severe infections (Santiago-Alarcon et al. 2016). Haemosporidian infections in birds can range from asymptomatic to severe, potentially leading to population declines or species extirpations. Juvenile and immunologically naive birds are at higher risk. Surviving individuals may act as reservoirs for transmission. Migratory birds often carry high parasite loads, which can infect native and captive species, especially in zoological settings (Meister et al. 2023). Diagnosis traditionally relies on blood smear examination or molecular techniques (El-Ghany 2023). Blood smear analysis may have low sensitivity in detecting low levels of parasitemia, requiring specialized training and expertise (Valkiūnas et al. 2008, Palinauskas et al. 2015). In contrast, molecular techniques can enhance diagnostic sensitivity, allow for the identification of cryptic species or lineages, and offer valuable insights into the genetic diversity of parasites (Morel et al. 2021). Advances in molecular techniques, particularly the analysis of the Cytochrome B (CytB) gene, have improved our understanding of parasite diversity (Bensch et al. 2000, Hellgren et al. 2007). The MalAvi database has been instrumental in summarizing parasite lineages and their distributions (Bensch et al. 2009).

Current research on avian haemoparasites, especially *Haemoproteus*, is limited, primarily focusing on passerines (Harl et al. 2022). We used both morphological and molecular techniques to enhance our understanding of haemosporidian parasites in wild raptors at the Progetto Natura Verona Lago ODV (Volunteer Organization, Wildlife Rehabilitation and Recovery Center). By focusing specifically on raptors, the research addresses knowledge gaps regarding the diversity and distribution of haemosporidian parasites.

METHODS

Sample Collection and Morphological Identification. We analyzed 47 blood samples: 37 from Eurasian Kestrels (*Falco tinnunculus*) and 10 from Eurasian Buzzards (*Buteo buteo*), collected between 2022 and 2023 at a rehabilitation facility in northern Italy (Progetto Natura Verona ODV, 45.49184°N, 10.76031°E). The reason for admission was recorded for each raptor, and blood samples (0.5 ml) were collected. All birds were admitted from rescue activities carried out in Verona and its province, a city located in the Veneto region of northern Italy, through the Verona Lago ODV. Blood samples were collected shortly after the birds' admission to the center, during the initial veterinary examination (generally within 24–48 hr of arrival). Although the majority of admissions were unrelated to parasitic infections, a few cases were associated with parasitic diseases, including *Capillaria*, coccidia, and *Trichomonas* infections. The main causes of admission were unknown causes, trauma—particularly from vehicle collisions, impact with power lines, and other anthropogenic factors—physical injuries such as fractures or cutaneous wounds, infectious diseases, poisoning or shooting (rare cases), grounding of nestlings or fledglings unable to survive on their own, predation by domestic animals, and in some instances, birds that arrived already dead.

We assessed blood samples using two diagnostic methods: microscopic examination of Giemsa-stained blood smears and sequencing of a fragment of the mitochondrial CytB. Blood samples for molecular analysis were stored at −20°C in 1.5 mL tubes. Blood smears were prepared on glass slides,

stained with Giemsa stain, and examined under a microscope for haemoparasites. The parasites identified were classified according to keys (Valkiūnas and Iezhova 2022).

DNA Isolation, Amplification, and Sequencing. Besides the 47 blood samples collected in the recovery facility, we added one Eurasian Hobby (*Falco subbuteo*) blood sample and one *Gallus gallus domesticus* hatchling blood sample to the dataset as positive and negative controls, respectively, to assess the validity of the experimental protocol. In addition, we randomly chose three samples to analyze twice independently—from sampling to sequencing—to test the repeatability of the molecular assessment. Total DNA was extracted from 52 blood (47 raptor samples, positive and negative controls, and the three replicates) spots using the Wizard Genomic DNA Purification Kit (Promega, Madison, Wisconsin, USA) following the company's procedure and immediately amplified according to the nested protocol developed for the screening of the mitochondrial CytB gene in *Plasmodium*, *Haemoproteus*, and *Leucocytozoon* parasites (Hellgren et al. 2004). The primer couple HaemNFI/HaemNR3 (Hellgren et al., 2004) was used in the first polymerase chain reaction (PCR). The first PCR product (1 µl) was used as a template in two different nested PCRs: nested mix 1 containing the primer couple HaemF/HaemR2 (Bensch et al. 2000) for *Plasmodium* spp. and *Haemoproteus* spp.; nested mix 2 containing the primer couple HaemFL/HaemR2L for *Leucocytozoon* spp. (Hellgren et al. 2004). In addition, DNA was also amplified according to a nested protocol (Harl et al. 2022) to identify the possible presence of parasites belonging to *L. toddi* group, using primer couple CytB_L2_F/CytB_L2_R in the first PCR and CytB_L2_nF/CytB_L2_nR in the nested PCR. Positive products at nested PCRs were purified by ReliaPrep DNA Clean-Up and Concentration System (Promega) and then sent to StarSEQ (Germany) for sequencing of both strands.

Bioinformatic Analyses. The obtained sequences were aligned by the ClustalW algorithm (Thompson et al. 1994) implemented in MEGA11 (Tamura et al. 2021); primer regions were removed and submitted to the Basic Local Alignments Search Tool (BLAST) algorithm both in National Center for Biotechnology Information (NCBI; doi:10.1002/cpet.8) and in MalAvi databanks for species identification. Orthologous sequences belonging to different haemosporidian genera were included in the dataset as a reference for bioinformatic analyses. Specifically, we added sequences belonging to the genera *Haemoproteus* (GenBank accession number EF564176, MK580170, MK580171, MN780908, MN780909, EF564177, MT281481, KC994901), *Parahaemoproteus* (GQ141621, GQ141558), *Leucocytozoon* (AY393796, EF607293, DQ177253), and *Plasmodium* (AY099029) as an outgroup. DNA statistics were computed using DnaSP 6.12.03 (Rozas et al. 2017). After the identification of the best-fitting model, we built a phylogenetic tree using the maximum likelihood method (Felsenstein 2004) implemented in MEGA11 (Tamura et al. 2021). We assessed the robustness of tree topology by the bootstrapping method with 1000 replicates (Felsenstein 1985).

Statistical Analysis. We used Fisher exact tests to compare the infection rates of the two raptor species overall, and the infection rates as determined by the molecular and haematological methods. We used Stata12 software for statistical analysis.

RESULTS

Morphological Identification. Hematological evaluation was not possible for three samples (two kestrels and one buzzard) due to cellular deterioration. The remaining 44 samples represented 35 kestrels and 9 buzzards. Of the 44 blood smears analyzed, 22 tested negative for the presence of haemoparasites, while the remaining 22 (16 kestrels and 6 buzzards) were positive (Table 1; Supplemental Material Table S1).

Eurasian Kestrels. We identified only *Haemoproteus* sp. in 15 out of the 35 kestrel samples (42.9%). One kestrel sample was positive for both *Haemoproteus* sp. and *Leucocytozoon* sp. (2.9%). Overall prevalence of *Haemoproteus* sp. in kestrels was 45.7% and that of *Leucocytozoon* sp. was 2.9%.

Eurasian Buzzards. The nine samples were infected as follows: two by *Haemoproteus* sp. only (prevalence 22.2%), three by *Leucocytozoon* sp. only (33.3%), and one with both genera (11.1%). The overall prevalence of *Haemoproteus* sp. in buzzards was 33.3%, while that of *Leucocytozoon* sp. was 44.4%.

Molecular Identification. We performed molecular analysis on all 47 available samples, including the 44 samples previously described as well as the three that were too degraded for morphological examination (CK1328, CK1526, and CB1347). Amplification of a 479 bp fragment of the CytB gene revealed 89 polymorphic sites, which defined three distinct lineages with a haplotype diversity Hd = 0.462. The most frequently detected lineage corresponded to *Haemoproteus brachiatus* belonging to the MalAvi lineage LK03 (GenBank accession numbers MK580170 and MT281478; Valkiūnas et al. 2019, Huang et al. 2020). This lineage was the most prevalent, with an overall frequency of 46.8%.

Table 1. Overview of morphological and molecular analyses showing the number of positive cases with the infection prevalence in parentheses. The sum of positives is larger than the examined number due to the presence of double infections, as detailed in the main text.

	Morphological Identification			Molecular Identification		
	Total	Kestrel	Buzzard	Total	Kestrel	Buzzard
Number examined	44	35	9	47	37	10
Infected	22 (50.0%)	16 (45.7%)	6 (66.7%)	27 (57.4%)	18 (48.6%)	9 (90.0%)
Haemoproteus	19 (43.2%)	16 (45.7%)	3 (33.3%)	22 (46.8%)	18 (48.6%)	4 (40.0%)
Leucocytozoon	5 (11.4%)	1 (2.9%)	4 (44.4%)	8 (17.0%)	0 (0%)	8 (80.0%)

The second lineage was identified as *Leucocytozoon* sp. MalAvi lineage BUBT2, with a frequency of 17.02%. The third lineage, *Haemoproteus* sp. MalAvi lineage BUTBUT04 (GenBank accession MT281481; Huang et al. 2020) was detected only in the positive control Eurasian Hobby. All the obtained sequences were submitted to GenBank under the following accession numbers: PV342350–PV342371 for *H. brachiatus* LK03 (22 sequences), PV342372–PV342380 for *Leucocytozoon* sp. BUBT2 (9 sequences), and PV342381 for the single *Haemoproteus* sp. BUTBUT04 sequence. The Maximum Likelihood tree showed a clear separation of the three lineages found (Fig. 1), two of which belonged to the *Haemoproteus* clade (LK03 and BUTBUT04) and one to the *Leucocytozoon* clade (BUBT2). The three replicated samples (CK126bis, CK651bis, and CK1255bis) confirmed the results of the initial analyses.

Eurasian Kestrels. In kestrels, only *H. brachiatus* LK03 was detected, with a prevalence of 48.6% among the 37 individuals analyzed. No other haemoparasite lineages were identified.

Eurasian Buzzards. *H. brachiatus* LK03 was identified in 40% of the Eurasian Buzzard samples, while *Leucocytozoon* sp. BUBT2 was detected in 80%. Three

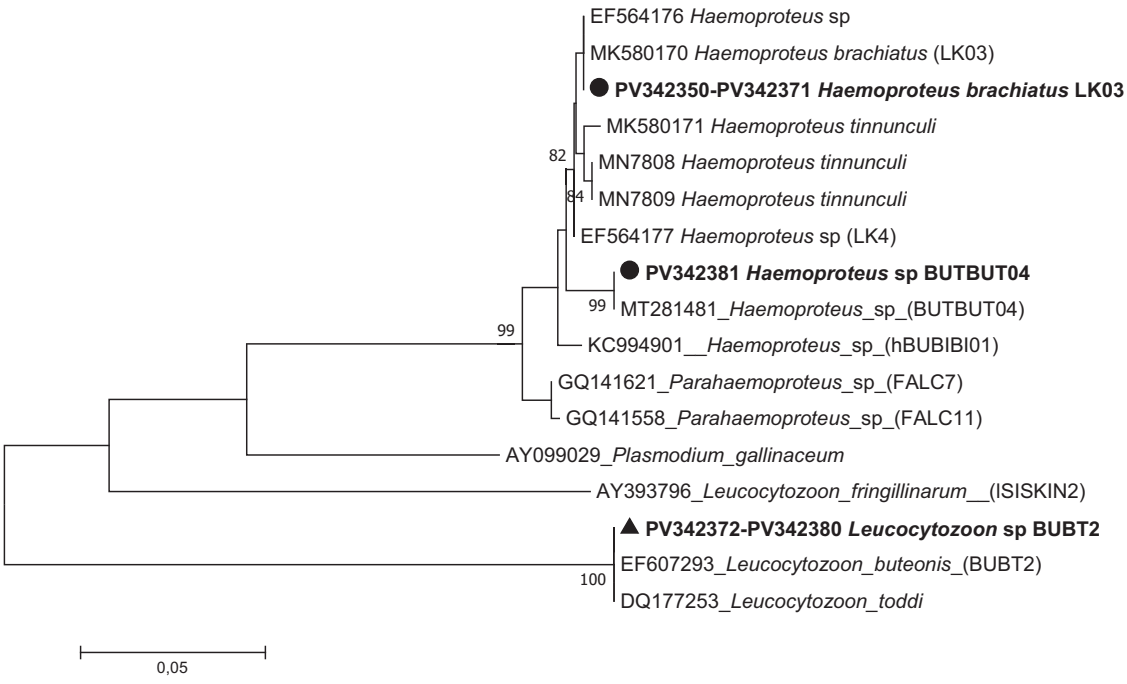

Figure 1. Maximum Likelihood tree. Numbers are bootstrap values, black dots represent the two main lineages found for *Haemoproteus* genus (LK03, and BUTBUT04), while the black triangle represents the single lineage found for *Leucocytozoon* genus (BUBT2).

Table 2. Contingency table comparing the results of morphological and molecular analyses for *Haemoproteus* parasite.

	Haemoproteus	
	Molecular Identification Positive	Molecular Identification Negative
Morphological identification positive	16	3
Morphological identification negative	5	20

buzzards were coinfected with both lineages (CB1347, CB14, and CB1548). For these three doubly infected birds, morphological examination of two samples (CB14 and CB1548) had revealed only *Leucocytozoon* spp., with no morphological evidence of *H. brachiatus*. The third sample (CB1347) could not be evaluated morphologically due to the poor quality of the blood smear.

Diagnostic Performance of the Two Protocols and Species Comparisons. Considering the molecular protocol as the criterion standard, the morphological protocol showed a sensitivity of 84.6% and a specificity of 100%. Table 2 and Table 3 summarize the diagnostic performance by parasite genus. For *Haemoproteus*, the morphological protocol showed a sensitivity of 76.2% and specificity of 86.9%, whereas for *Leucocytozoon*, sensitivity was 57.1% and specificity was 97.3%.

Eurasian Buzzards at the rehabilitation center were significantly more infected than Eurasian Kestrels (Fisher's exact test, $P = 0.029$). Although overall haemoparasite prevalence observed through the morphological protocol did not differ significantly between the two species (Fisher's exact test, $P = 0.73$), the molecular protocol detected a higher prevalence of *Leucocytozoon* in buzzards compared to kestrels (Fisher's exact test, $P < 0.0001$).

DISCUSSION

Although haemosporidian parasites are cosmopolitan, frequently detected in birds, and potentially capable of causing severe damage to their hosts, they remain partly neglected due to the challenges of species identification using morphological methods alone. These difficulties hinder comprehensive research on parasite diversity. Molecular studies have contributed to partially filling this gap, describing over 4600 unique CytB lineages, for a total of more than 170 different species identified, with many more likely to exist (Valkiūnas and Iezhova 2022).

Considering the molecular protocol as the criterion standard, we identified the presence of *Haemoproteus brachiatus* in both Eurasian Kestrels and Eurasian Buzzards from northern Italy, and also *Leucocytozoon* spp. in buzzards. No *Plasmodium* spp. was detected in any sample, which was consistent with published reports (Pornpanom et al. 2021).

Morphological Identification and Comparison with Molecular Results. *Eurasian Kestrels.* In Eurasian Kestrels, our findings confirmed the presence of *Haemoproteus* parasites with morphological features consistent with the molecular identification of *H. brachiatus*. However, the morphological similarity between haemosporidian species, as well as the difficulty in detecting low-intensity infections, limits the reliability of microscopy alone. This is supported by two specimens in our study that were negative by morphological examination, but tested positive by molecular analysis.

Eurasian Buzzards. In Eurasian Buzzards, both *Haemoproteus* spp. and *Leucocytozoon* spp. were detected morphologically. Two birds (one kestrel and one buzzard) tested positive for both *Leucocytozoon* and *Haemoproteus* by microscopy, but only for one parasite by molecular analysis (*Haemoproteus* in the kestrel and *Leucocytozoon* in the buzzard). These discrepancies might represent false positives in morphological identification, possibly due to atypical forms mimicking other haemoparasite genera.

However, the possibility of false negatives by molecular analysis, especially under low parasitemia conditions, cannot be excluded. In two other

Table 3. Contingency table comparing the results of morphological and molecular analyses for *Leucocytozoon* parasite.

	Leucocytozoon	
	Molecular Identification Positive	Molecular Identification Negative
Morphological identification positive	4	1
Morphological identification negative	3	36

buzzard samples (CB14 and CB1548), molecular analysis revealed coinfections with both *Haemoproteus* and *Leucocytozoon*, while microscopy detected only *Leucocytozoon*. These cases likely represent false negatives in morphological diagnosis due to the difficulty of detecting low-intensity *Haemoproteus* infections. Our findings highlight the limitations of morphology-based diagnosis alone and the usefulness of combining morphological and molecular approaches for reliable identification.

Molecular Identification and Lineage Analysis. *Eurasian Kestrels.* In Eurasian Kestrels, the identification of the single genetic lineage (LK03) suggested that all the *Haemoproteus* infections in this study originated from a single strain (or from closely related strains) within the northern Italian kestrel population.

Eurasian Buzzards. A single *Leucocytozoon* genetic lineage was identified in all nine positive buzzard samples (BUBT2), suggesting that, as with *Haemoproteus* infections in both hosts, the *Leucocytozoon* infections in buzzards likely originated from a single strain circulating within the northern Italian buzzard populations.

The prevalence of haemosporidian infections in Eurasian buzzards in our study (90% overall) was higher than values reported in Iranian buzzards (54.5%; Shokrani et al. 2021) and German buzzards (31%; Krone et al. 2001). The Iranian study identified *Haemoproteus* at the lineage level (BUTBUT15), whereas the German study relied on morphological identification without molecular lineage characterization. Therefore, while these comparisons are valid at the genus level, differences in parasite lineages cannot be excluded and may contribute, together with geographical and methodological factors, to the observed variation in prevalence.

Host Specificity, Geographic Variation, and Diagnostic Consideration. The detection of *H. brachiatus* in both host species, and of *Leucocytozoon* exclusively in buzzards, confirmed the presence of haemosporidian parasites with different host-specificity. All *Haemoproteus* isolates belonged to lineage LK03, and all *Leucocytozoon* to BUBT2, indicating low intraspecific diversity. This uniformity may reflect adaptation to specific host or vector species and has implications for understanding parasite transmission dynamics within and among host populations. For example, on the arid Cape Verde Islands, Hille et al. (2007) reported a very low prevalence (0.8%) of *Haemoproteus brachiatus* in 130 kestrels sampled. This result was likely influenced by the low vector density typical of such an arid environment, as well as by the exclusive use of microscopy, which may have failed to detect low-intensity infections. In contrast, Korpimäki et al. (1995) reported intermediate prevalence for *Haemoproteus tinnunculi* (40% in females, 25% in males) and *H. brachiatus* (13% in females, 10% in males) among kestrels in Finland.

Benefits and Challenges of Using Molecular Identification. In our study, the use of both microscopy and nested PCR followed by Sanger sequencing allowed detection of infections with higher sensitivity and revealed mixed infections not visible in blood smears. However, the presence of cross-amplification between primer couples specifically designed to distinguish between *Haemoproteus* and *Leucocytozoon* genera, and between *L. toddi* and other *Leucocytozoon* species, suggests that in the absence of the main target DNA, they might bind to non-target DNA regions, leading to false positives for a given genus or species. This result suggests that nested PCR alone sometimes is not sufficient, and the sequencing step is mandatory. In the present study, the presence of *Haemoproteus nisi/multivacuolatus* might have been underestimated, as standard primers by Hellgren et al. (2004) do not efficiently amplify these lineages. Newly developed primers (Harl et al. 2022) may improve detection of these parasites, especially in Eurasian Buzzards and other Accipitriformes.

Conclusions. Our survey revealed a unique lineage of *Leucocytozoon* sp. in buzzards and a unique lineage of *Haemoproteus brachiatus* both in buzzards and in kestrels, suggesting the circulation of this latter parasite among a community of raptor host species, whereas *Leucocytozoon* seems to be more specific. In addition, the different performance of molecular and morphological diagnosis suggests the need for the development of new molecular diagnostic protocols. As a caveat, our findings should be interpreted in light of certain methodological limitations, including moderate sample size and potential underestimation of diversity due to primer design. Future studies using improved markers and broader sampling are needed to confirm and extend our results.

SUPPLEMENTAL MATERIAL (available online). Table S1: Overview of morphological and molecular data available for each sample analyzed.

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