

Prevalence of Middle East Respiratory Syndrome Coronavirus in Dromedary Camels, Tunisia

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Free-roaming camels, especially those crossing national borders, pose a high risk for spreading Middle East respiratory syndrome coronavirus (MERS-CoV). To prevent outbreaks, active surveillance is necessary. We found that a high percentage of dromedaries in Tunisia are MERS-CoV seropositive (80.4%) or actively infected (19.8%), indicating extensive MERS-CoV circulation in Northern Africa.

Middle East respiratory syndrome (MERS) coronavirus (MERS-CoV) has the highest lethality of all known human coronaviruses; the case-fatality rate is 34.3% (1). The virus, first isolated in Saudi Arabia in 2012 (2), most likely originates from bats (3). However, several studies suggest that the zoonosis is mainly transmitted to humans by dromedary camels (*Camelus dromedarius*) (4–6).

As of January 2020, a total of 2,519 human MERS-CoV infections and 866 related deaths had been reported to the World Health Organization from 27 countries. Most of these cases (84.2%), including 788 related deaths, occurred in Saudi Arabia (1).

Given the close trading links between the Middle East and Africa, the risk for transferring the zoonosis is high. Tunisia is not a popular trading location, which makes undocumented transfers of dromedary camels within the country or with neighboring countries difficult to track. Only 3 human MERS cases have been imported from Qatar (7), and no autochthonous MERS-CoV infections have been reported for Tunisia. However, severe underestimation of human

MERS cases is highly probable because of the broad range of manifestations, from asymptomatic infection to acute pneumonia.

Furthermore, epidemiologic surveillance of MERS-CoV is limited in Tunisia, and no respective data for neighboring countries is publicly available. Two studies analyzing MERS-CoV prevalence in dromedaries in Tunisia reported high seropositivity of the sampled animals (49.0% and 87.3%) (8,9). However, those studies analyzed dromedary camels from livestock markets, slaughterhouses, and meat farms, without representing natural habitats. This limitation complicates drawing realistic conclusions about geographic and age-dependent distributions. We investigated the prevalence of MERS-CoV in dromedary camels in Tunisia primarily by sampling animals that roam freely through the desert during summer to determine an authentic representation of the distribution pattern.

The Study

Winter is mating and birthing season for dromedary camels. Therefore, animals kept for milk and meat production gather in areas that provide access to salty plants and other minerals. This environment provides an optimal opportunity to catch and examine large numbers of animals from different herds and origins, given that the camels that roam freely through the desert the rest of the year congregate simultaneously.

In January 2020, we collected serum samples and nasal swabs of 382 gathered animals in Tunisia. We sampled an additional 119 camels used for transport or patrol purposes, all of which were males and kept enclosed. The specimens were collected from 20 different locations within the Kebili Governorate (Table; Figure 1, panel A). Furthermore, serum samples of 22 camel keepers and 2 veterinarians were obtained. We

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have compiled details of our sampling and testing methods (Appendix, <https://wwwnc.cdc.gov/EID/article/27/720-4873-App1.pdf>).

We analyzed all 501 dromedary serum samples for MERS-CoV-specific antibodies by ELISA and found 80.4% to be seropositive for MERS-CoV IgG. At 85.7%, MERS-CoV seropositivity was higher in female than in male camels (65.6%) (Table; Figure 1, panel A).

Although none of the calves (0–6 months of age) and 4.9% of the juvenile camels (6–24 months of age) were seropositive for antibodies against MERS-CoV, relative seropositivity increased with age (Table). None of the camel keepers or veterinarians was seropositive, indicating no previous MERS-CoV infection.

Screening the dromedary nasal swab specimens for active virus infections with real-time reverse tran-

scription PCR revealed MERS-CoV RNA in 19.8%. However, cycle thresholds >30 (for all but 6 samples) indicated low virus concentrations. Female animals (23.0%) actively shed MERS-CoV RNA, whereas only 10.7% of the male camel specimens were PCR-positive. In contrast to the immunologic findings, a high percentage (40%) of juvenile camels (<2 years of age) shed MERS-CoV RNA, compared with 17.8% of the adult camels that tested positive (Table, Figure 1, panel A).

In summary, 433 of 501 dromedaries tested positive for MERS-CoV. A total of 334 animals were seropositive for MERS-CoV IgG but did not shed MERS-CoV RNA. Of these, 30 dromedary swab specimens contained MERS-CoV RNA, but no specific antibodies were found in the respective serum samples. Sixty-nine PCR-positive dromedary camels also had

Table. MERS-CoV IgG seropositivity and viral RNA presence in dromedary camels, by selected sampling parameters, Tunisia*

Sampling parameter	No. dromedaries	ELISA serologic testing, no. (%) positive for MERS-CoV IgG	Molecular biology rRT-PCR, no. (%) positive for MERS-CoV RNA
Sex			
M	131	86 (65.6)	14 (10.7)
F	370	317 (85.7)	85 (23.0)
p value		<0.05	<0.01
Age group			
Juvenile	45	2 (4.4)	18 (40.0)
0–6 mo	4	0 (0)	1 (25.0)
6–24 mo	41	2 (4.9)	17 (41.5)
Adult	456	401 (87.9)	81 (17.8)
2–6 y	81	62 (76.5)	19 (23.5)
6–12 y	179	157 (87.7)	28 (15.6)
12–25 y	190	176 (87.9)	32 (16.8)
>25 y	6	6 (100)	2 (33.3)
p value, juvenile compared with adult		<0.00001	<0.01
Sampling site			
Ksar Ghilane, n = 6	211	154 (73.0)	49 (23.2)
Site 1	28	20 (71.4)	7 (25.0)
Site 2	20	8 (40.0)	0 (0)
Site 3	30	26 (86.7)	6 (20.0)
Site 4	20	19 (95.0)	1 (5.0)
Site 5	73	50 (68.5)	25 (34.2)
Site 6	40	31 (77.5)	10 (25.0)
Bazma, n = 7	168	152 (90.5)	32 (19.1)
Site 1	25	24 (96.0)	2 (8.0)
Site 2	30	25 (83.3)	8 (26.7)
Site 3	15	13 (86.7)	3 (20.0)
Site 4	15	14 (93.3)	1 (6.7)
Site 5	21	20 (95.2)	4 (19.0)
Site 6	16	13 (81.3)	6 (37.5)
Site 7	46	43 (93.5)	8 (17.4)
Douz, n = 5	53	32 (60.4)	4 (7.5)
Site 1a	4	3 (75.0)	0 (0)
Site 1b	4	0 (0)	0 (0)
Site 2	24	18 (75.0)	3 (12.5)
Site 3	18	10 (55.6)	1 (5.6)
Site 4	3	1 (33.3)	0 (0)
Mahrouga, n = 2	69	65 (94.2)	14 (20.3)
Site 1	42	40 (95.2)	3 (7.1)
Site 2	27	25 (92.6)	11 (40.7)
p value for comparisons among all 4 main sites		0.05	Not significant
Total	501	403 (80.4)	99 (19.8)

*MERS-CoV, Middle East respiratory syndrome coronavirus; rRT-PCR, real-time reverse transcription PCR.

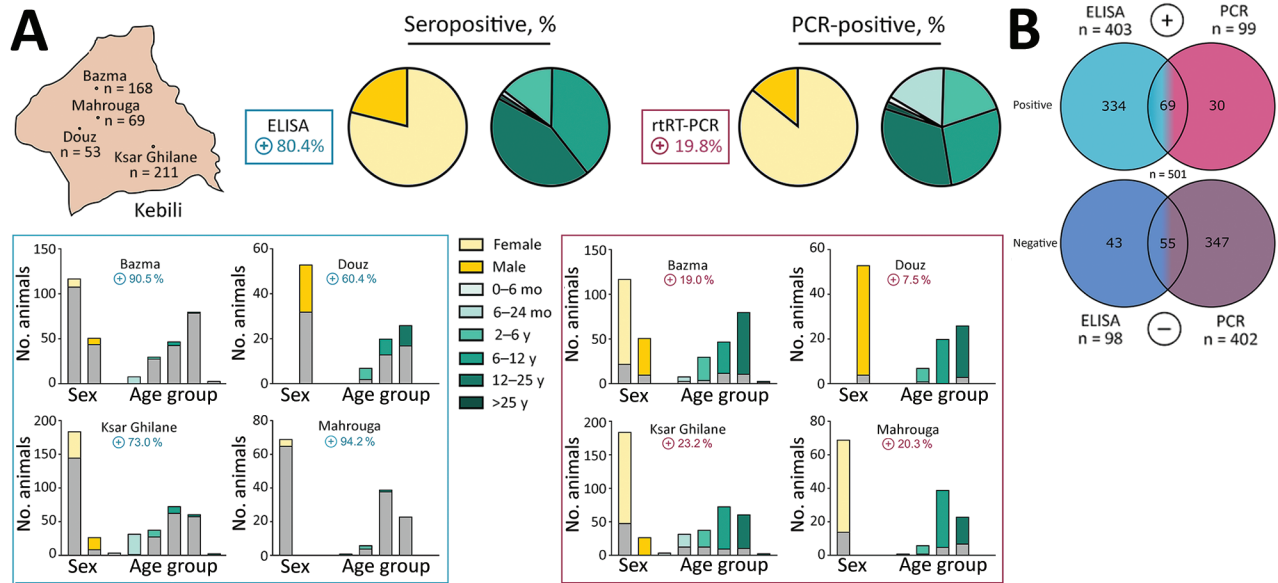


Figure 1. MERS-CoV prevalence in dromedary camels, Tunisia. A) Overview of seropositive and PCR-positive dromedaries from 4 different sampling areas (Appendix, <https://wwwnc.cdc.gov/EID/article/27/720-4873-App1.pdf>), by sex and age group. Blues boxes represent seropositive dromedaries and purple boxes PCR-positive dromedaries. Bars in chart represent total number of sampled animals; gray-shaded bar sections represent total number of MERS-CoV-positive animals. In the Douz area, all dromedary camels sampled were used for patrol or transport purposes and were exclusively adult male animals. **B)** Distribution of ELISA (blue) and PCR (purple) results, by number. Bright colors represent positive results, dark colors negative results; 2-colored areas represent animals that are either positive for both ELISA and PCR or negative for both. MERS-CoV, Middle East respiratory syndrome coronavirus;

MERS-CoV antibodies, indicating reinfection (Figure 1, panel B).

Attempts to cultivate the virus from all respective PCR-positive swab specimens were unsuccessful, most likely because of the low virus concentrations in the samples. Presumably, whole-genome sequencing did not work for the same reasons. However, we performed Sanger sequencing of cDNAs obtained from PCR-positive samples with the highest viral concentrations and subsequently conducted phylogenetic analysis with a 720-bp fragment of the spike receptor-binding protein. The analyzed nucleotide sequences from the dromedaries in Tunisia differ from previously published MERS-CoV sequences and therefore form a separate group distinct from strains found in Arabia. Two MERS-CoV isolates in Egypt, however, cluster in the same clade (Figure 2).

Conclusions

On the continent of Africa, active surveillance, longitudinal studies, and epidemiologic monitoring are scarce, and little is known about the prevalence and circulation of MERS-CoV in many regions. Whether MERS-CoV lineages in Africa have a lower tendency to cross the species barrier and infect humans is not fully understood. Therefore, closing

the gaps in surveillance and virus prevalence data remains a focus for all regions with dromedary camel populations.

Seroprevalence studies in Egypt, Ethiopia, Nigeria, and Kenya all indicate MERS-CoV circulation within camel herds, reporting seropositivity rates ranging from 30% to 100% (10). For dromedaries in Tunisia, only 2 studies have been published, reporting 49% and 87.3% MERS-CoV seropositive animals and only 0.7% active viral shed (8,9).

However, most studies focus on locations where large numbers of camels congregate (e.g., abattoirs, large-scale farms, harbors, or livestock markets). At these locations, dromedaries are kept at a substantially increased population density compared with their normal habitats. This practice, referred to as crowding, increases stress for individual animals (11). Under these circumstances, increased intensive animal contact can lead to higher transmission rates of various microorganisms. Crowding, in combination with animal transport, is known to promote infections of the upper and lower respiratory tract, especially in cattle (12). In contrast, the camels investigated in our study represent a rare example of MERS-CoV prevalence in animal groups with a natural herd structure in northern Africa.

We found extensive MERS-CoV IgG seropositivity (80.4%) and high ratios of MERS-CoV RNA (19.8%) among dromedaries in Tunisia. Compared with adult animals, juvenile camels were more likely to have active MERS-CoV infections and less MERS-CoV IgG in their serum samples. Furthermore, some dromedaries

appeared to have MERS-CoV reinfections, explained by the fact that coronaviruses tend to establish endemic infection patterns with high seroprevalence and low but continuous viral shedding in their natural host (13). Waning antibodies in combination with antigenic drift of the virus fosters reinfection events (14).

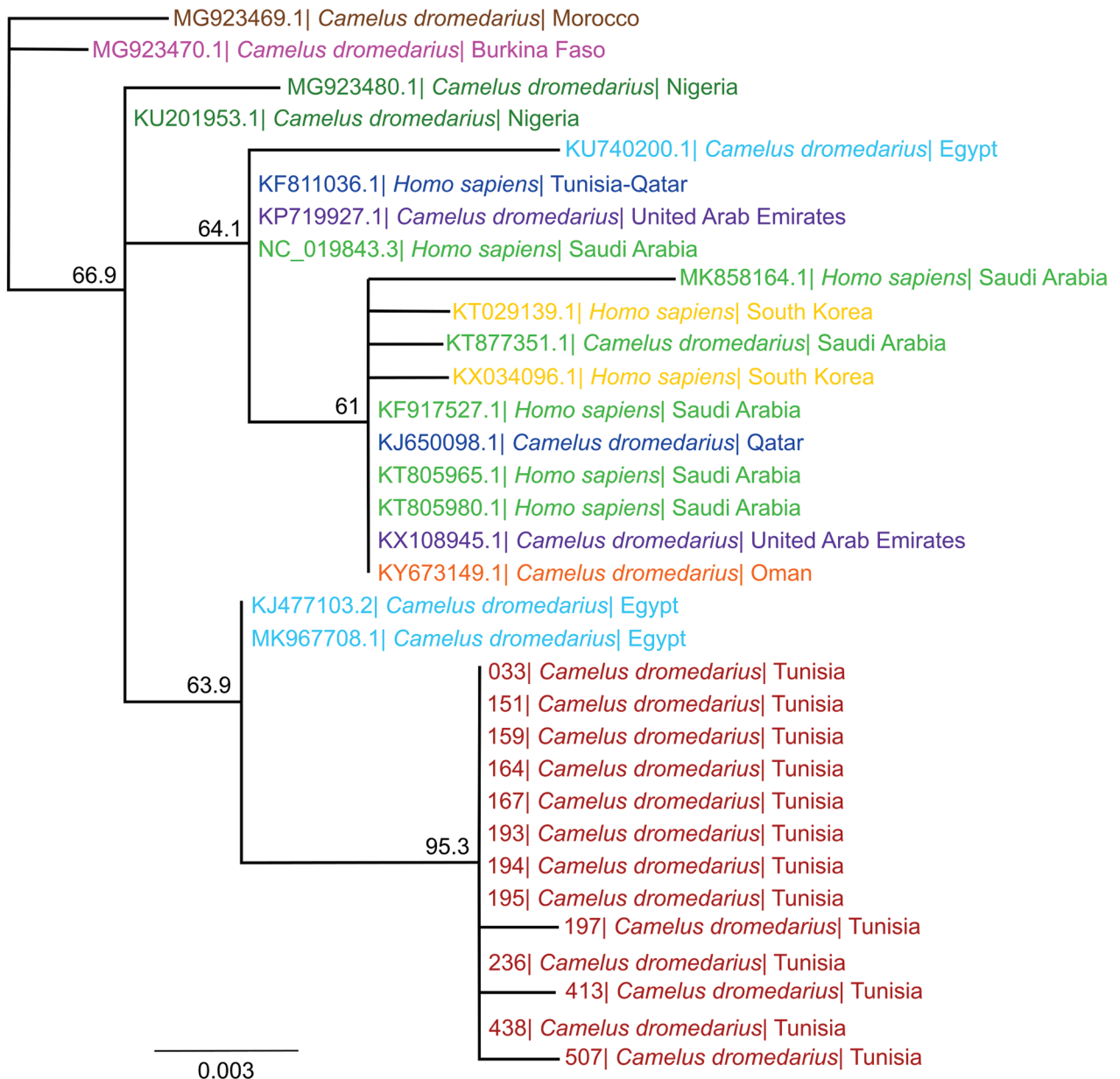


Figure 2. Phylogenetic analysis of MERS-CoV samples from dromedary camels in Tunisia, conducted by using the spike RBD. We used 720-bp fragments of the MERS-CoV spike RBD amplified from nasal swab samples of 13 dromedary camels and published RBD sequences of representative MERS-CoV strains from other countries to create the phylogenetic tree using Geneious Prime Tree Builder (Geneious Biologics, <https://www.geneious.com>). Branches are shaded by country: red represents sequences from Tunisia (this study); brown represents Morocco, pink Burkina Faso, dark green Nigeria, blue Egypt, dark blue Qatar, green Saudi Arabia, yellow South Korea, purple United Arab Emirates, and orange Oman. GenBank accession numbers are provided for reference sequences. Numbers indicate bootstrap values (1,000 pseudo-replicates). Scale bar indicates sequence divergence (% nucleotide substitutions). MERS-CoV, Middle East respiratory syndrome coronavirus; RBD, receptor-binding protein.

A limitation of our study is that the low sample size of humans tested, comprising 22 camel keepers and 2 veterinarians (data not shown), precludes drawing generalized conclusions. Furthermore, no nasal swab specimens were collected from camel keepers to check for active MERS infections. Also, no phenotypic or whole-genome analysis of MERS-CoV strains from the dromedary camels was possible because virus growth and next-generation sequencing were not successful because of low viral concentrations.

In conclusion, the high seroprevalence of MERS-CoV antibodies and the active shed of MERS-CoV RNA indicate the widespread nature of the virus in dromedaries in Tunisia. However, more extensive studies in the human and dromedary camel populations and in-depth whole-genome sequence analysis of circulating MERS-CoV strains are required to increase epidemiologic understanding of the disease and its infection dynamics.

Acknowledgments

We thank the Deutsche Gesellschaft für Internationale Zusammenarbeit for their support by planning and organizing the study. Furthermore, we gratefully acknowledge the camel keepers in Tunisia for their participation.

This work is part of Medical Biological Defense Research Program of the Bundeswehr Medical Service and was supported by the Enable and Enhance Initiative of the federal government of Germany (Ertüchtigungsinitiative der Deutschen Bundesregierung, grant no. OR12-370.43 ERT TUN IMB).

About the Author

Dr. Eckstein is a scientist at the Bundeswehr Institute of Microbiology. Her research interests include viral and bacterial zoonoses in North Africa.

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Appendix

Materials and Methods

Sampling

A total of 501 dromedary camels' nasal swabs as well as serum samples were collected at 20 different sites for Middle East Respiratory Syndrome Coronavirus (MERS-CoV) screening in January 2020. The individual sampling sites were in the surrounding areas of Ksar Ghilane (6 sites; n = 211), Douz (5 sites; n = 53), Bazma (7 sites; n = 168) and Mahrouga (2 sites; n = 69) within the Kebili governorate (Table 1, Figure 1A,). 382/501 samples were obtained from dromedary camels in their natural habitat at Ksar Ghilane (sampling site 1 and 3–6,) Bazma (sites 1–6) and Mahrouga (sites 1+2). Those herds kept for milk and/or meat production each consisted of one sultan, its female harem and some juvenile male animals. However, to avoid too high stress level for the animals, not all camels in a herd could be sampled. The remaining 119 camels, exclusively adult males used for transport or patrol purposes and therefore kept enclosed, were from around Douz (sites 1a+b-4), Ksar Ghilane (site 2) and Bazma (site 7).

A total of 131 male and 370 female dromedary camels ranging in age from juvenile (0–6 months: 4 camels and 6–24 months: 41) to adult (2–6 years: 81 camels; 6–12 years: 179; 12–25 years: 190 and >25 years: 6) were sampled.

Additionally, 22 camel keepers (men between the ages of 12 to 69) from the sampling sites in Ksar Ghilane and Douz) having daily contact with their dromedary camels as well as two veterinaries (men, 25 and 48 years) with frequent animal contact were willing to provide serum for investigation of previous MERS-CoV infections.

Immunoserological testing

Seroprevalence of anti-MERS-CoV IgG in dromedary camels as well as camel keepers was investigated by performing indirect ELISA assays (Euroimmun AG, Germany) according to the manufacturer's protocol.

Molecular testing

Viral RNA was extracted using the QIAamp Viral RNA Mini Kit (QIAGEN, Netherlands). Subsequently, real-time reverse transcription PCR (rtRT-PCR) was performed targeting the genetic region upstream of the *E* gene (*upE*) (for screening) and *ORF1a* (for confirmation), respectively, as described elsewhere (1,2).

Statistical analysis

Associations between MERS-CoV prevalence in dromedary camels and the study parameters (sex, age and sampling site) were analyzed by Pearson Chi-square test for comparison of categorical data. At a p-value of less than 0.05, statistical significance was considered.

Virus isolation in cell culture

Isolation of live virus was attempted on Vero E6 cells under BSL3 conditions. Briefly, 250 µl of virus transport medium of MERS-CoV rtRT-PCR-positive nasal swabs was used as inoculum for cell cultures in T25 flasks. Cells were washed with serum free medium, inoculated with sample fluid and incubated for one hour. Afterwards, the inoculum was removed and the cellular layer was washed three times. The final culture medium contained MEM with 2% FBS and 5x antibiotic-antimycotic additive (GIBCO, USA). Cells were incubated for 7 days with one subsequent subpassage of supernatant on fresh cells. Cultures were regularly screened for cytopathic effects and supernatant was screened for an increase in MERS-CoV RNA.

Genomic spike RBD amplification

For phylogenetic analysis a partial 774-bp fragment of the spike (*s*) gene containing the RBD was amplified by running a rtRT-PCR (SSIII qRT-Kit, Invitrogen, United States) using the primer pair pre-RBD fwd (GAATCTGGAGTTTATTCAGTTTCGT) and pre-RBD rev (ACGGCCCGAAACACCATAG) (3). Subsequently, spike RBD (720-bp) was amplified by performing a nested PCR with the primers RBD fwd (GAAGCAAAACCTTCTGGCT) + RBD rev (ATATTCCACGCA) using Q5 HotStart MasterMix Kit (New England Biolabs, USA) with

the respective pre-fragment as DNA template. The resulting PCR product was gel-purified (Mini kit for gel extraction and PCR clean up, Macherey-Nagel, Germany) and sequenced with the latter primers (Eurofins Genomics Germany GmbH, Germany).

Sequence analysis

The phylogenetic tree was constructed based on 13 s RBD (GenBank AcNo: MW322770-MW322782) sequences, obtained from rtRT-PCR-positive dromedary specimens with the highest viral amounts and representative MERS-CoV sequences from different countries using Geneious Prime (Version 2020 1.2) by applying the neighbor-joining method with Tamura-Nei's genetic distance model and 1,000 bootstrap replicates.

Ethics Statement

The use of the biologic material described in the underlying study was approved by the Ethics Committee of the Military Hospital in Tunis (Decision N° 57/2020/CLPP) and written consent forms were signed by all included participants.

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