MAJOR ARTICLE

Mycobacterium tuberculosis Complex DNA from an Extinct Bison Dated 17,000 Years before the Present

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In order to assess the presence of tuberculosis in Pleistocene bison and the origin of tuberculosis in North America, 2 separate DNA extractions were performed by 2 separate laboratories on samples from the metacarpal of an extinct long-horned bison that was radiocarbon dated at 17,870 ± 230 years before present and that had pathological changes suggestive of tuberculosis. Polymerase chain reaction amplification isolated fragments of tuberculosis DNA, which were sequenced, and on which spoligotyping was also performed to help determine its relationship to the various members of the *Mycobacterium tuberculosis* complex. Extensive precautions against contamination with modern *M. tuberculosis* complex DNA were employed, including analysis of paleontologic and modern specimens in 2 geographically separate laboratories.

Recognition of tuberculosis-compatible pathology in bones of North American Pleistocene bovids has suggested that tuberculosis was present from an early date on that continent. This is now confirmed by the results of DNA sequencing for a bone sample from a bison dated to 17,000 years before the present (BP). The presence of similar pathology in fossil bighorn sheep and musk ox suggests that *Mycobacterium tuberculosis* complex organisms were widespread in bovids that immigrated to North America over the Bering Strait connection and widespread during the late Pleistocene. It suggests Holarctic distribution and that bovids were the likely vector and reservoir for dispersion of what became known as the white plague.

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Clarification of the evolutionary history of infectious diseases affords great opportunity for understanding the factors that have created current epidemiological patterns. Study of pathognomonic lesions in fossils has allowed progress in this endeavor [1–3], but for many lesions a specific etiology cannot be determined. PCR and application of DNA spoligotyping and sequencing techniques [4–8] allow that limitation to be transcended, but the very sensitivity of PCR raises the additional challenge of contamination [7, 8]. Contamination of ancient by modern DNA is now sufficiently recognizable (and usually preventable) that confident diagnosis of ancient DNA is possible [7, 8].

The resurgence of tuberculosis in recent years emphasizes the current limits to its control [9, 10]. Knowledge of the evolutionary history and the antibiotic susceptibilities of the *M. tuberculosis* complex may provide insights that will lead to better control in the future. The issue is complex, however. The standard DNA probes for tuberculosis recognize what has been called the "*M. tuberculosis* complex" [11, 12], which consists

of *M. tuberculosis, Mycobacterium bovis, Mycobacterium africanum*, and *Mycobacterium microti* [13]. The term "*M. tuberculosis* complex" distinguishes this group of organisms from saprophytic mycobacteria (e.g., soil organisms) and atypical mycobacteria such as *Mycobacterium avium–intracellulare* [14].

Additional analysis is required to distinguish among the members of the *M. tuberculosis* complex. Spoligotyping clearly distinguishes modern *M. tuberculosis* from modern *M. bovis* and from non–*M. tuberculosis* complex mycobacteria [15].

Documentation of *M. tuberculosis* complex in humans on both sides of the Atlantic Ocean who lived >3 millennia ago [2, 16–18] has led to a great deal of speculation about its origins. Such work suggests that at least some of the previously reported presumptive diagnoses [16–30] were accurate. The presence of *M. tuberculosis* complex on both sides of the Atlantic at such early dates suggests that it either arrived with early settlers in North America or was present in North America when they arrived. The latter hypothesis is supported by suggestive evidence in the fossils of North American bovids [31].

One of the classic findings in tuberculosis is the undermined subchondral articular surface [3, 32]. That finding, which is relatively specific for the diagnosis of tuberculosis, is common in fossil bones from the Late Pleistocene in North America [31].

This study involved the use of molecular DNA techniques to identify the presence of *M. tuberculosis* complex in a skeletal specimen with lesions suggestive of tuberculosis from Natural Trap Cave (Wyoming). That unique site was an unavoidable hazard on a game trail, providing an unbiased sampling of the many passing species. Identification of the DNA fragments was done by spoligotyping and direct DNA sequence determination. Analysis was conducted at 2 separate laboratories (Department of Bacteriology, Royal Free Hospital and University College, London, and Department of Parasitology, Hebrew University, Jerusalem) to ensure the reproducibility of the findings and eliminate the possibility of contamination.

Our results provide the first definitive diagnosis of *M. tuberculosis* complex in a fossil. They suggest that bovids were the vectors that transported the primordial organism that caused what we today call the white plague: tuberculosis.

METHODS

Sample source. Bones were recovered in Wyoming from the Natural Trap Cave, a chamber nearly 30 m deep with a 4-m opening. The cave lies across an ancient game trail that leads to and from low-altitude pastures. For >100,000 years it has trapped animals such as bears, wolves, foxes, wolverines, martens, cheetahs, lions, horses, mammoths, camels, sheep, musk oxen, and bison [33]. The depth of the cave makes falls invariably fatal and also keeps the temperature nearly constant at 4°C–5°C.

During a survey of all of the approximately 40,000 bones recovered from Natural Trap Cave, a classic but relatively rare skeletal expression of granulomatous infection, involving undermining of subchondral surfaces, was noted in bovids (i.e., bighorn sheep, extinct musk ox, and bison). These lesions were absent in the very large horse and American pronghorn antelope samples from that site [31]. Radiological analysis revealed peri-erosive osteopenia (decrease in the bone density around the involved area). The most likely etiologic agent of this paleopathology was *M. tuberculosis*.

Samples and controls. Two separate samples were taken from the undermined articular surface region of a metacarpal of an extinct long-horned bison *(Bison* cf. *antiquus)* that was enclosed in sediments dated by radiocarbon analysis at 17,870 ± 230 years BP. Areas distinct from the pathological lesion were also examined. Fossil controls consisted of lesion-free bones from *Canis* species and *Equus* species.

DNA extraction. Two separate DNA extractions were independently analyzed by 2 geographically separate laboratories. Cell lysis with guanidinium thiocyanate and DNA capture onto silica was used on nondecalcified samples in the Jerusalem laboratory and on decalcified samples in the London laboratory [34–36]. Stringent precautions were taken at both laboratories to avoid cross-contamination. DNA extraction and PCR were performed with standard precautions, including the use of ultraviolet-irradiated safety cabinets, ultrapure reagents, and sterile disposables under full isolation procedures. Control samples that contained no bone material and bone material from nonlesional regions and from unaffected species were used to assess contamination during the extraction and amplification processes.

DNA amplification. Different PCR systems were used in the 2 laboratories. The Jerusalem laboratory used primers from the ribosomal protein S12 [5, 6] to amplify a 204-bp DNA fragment: forward 5′-TCGTCGGGACAAGATCAGTAAG-3′ (position 33–54) and reverse 5′-ATCGAGTGCTCCTGCAGGTTG-3′ (position 216–236). In addition, a primer based on the insertion sequence IS6110 [37] was also used to amplify a region of 245 bp: forward 5′-CGTGAGGGCATCGAGGTGGC-3′ (position 633–652) and reverse 5′-GCGACGTAGGCGTCGGTGACAAA-3′ (primer position 880–858).

The London laboratory used primers to amplify a smaller segment from within the IS6110. A 2-stage nested PCR was used: the first reaction amplified a 123-bp region of IS6110 [14], and a second nested reaction amplified a 92-bp inner segment [38]. The latter segment was as follows: TTCGGACCACCAGCACCTAA (bases 777–796 of X17398, aka IS3) and TCGGTGACAAAGGCCACGTA (bases 868–849 of X17398, aka IS4).

Amplification was performed in a solution of 20 m*M* of Tris-HCL (pH, 7.5), 40 m*M* of NaCl, 2 m*M* of sodium phosphate, 0.1 m*M* of EDTA, 1 m*M* of dithiothreitol stabilizers (Sigma-Aldrich), 50% (v/v) glycerol, 1.5 m*M* of $MgCl_2$, 40 p*M* of each primer, 0.2 m*M* each of nuclear triphosphates (MBI, Fermentas), and 1.25 U of Platinum Taq DNA polymerase (GibcoBRL Life Technologies). Amplification consisted of initial denaturation at 94°C for 2 min, followed by 39 cycles of 94°C for 30 s, 61°C for 45 s, 1 min at 72°C, and, finally, 10 min at 72°C.

DNA sequence analysis. The Jerusalem laboratory performed direct sequencing using the Termo-Sequenase kit (Amersham), followed by agarose gel electrophoresis of the PCR product. The band was cut from Nu-Sieve low-melting-point agar and used as such in the labeling mix. The London laboratory removed the selected band from the gel and extracted it using a MERmaid Spin Kit (10–200 nucleotides; Anachem). The purified DNA was then fluorescence-labeled with the ABI PRISM Dye Terminator Cycle sequencing kit with AmpliTaq DNA Polymerase, FS (PE Applied Biosystems). The sequence was analyzed in an ABI PRISM 310 automated sequencer (PE Applied Biosystems).

Spoligotyping. To determine the species of *Mycobacterium* within the *M. tuberculosis* complex, the spoligotyping system (Isogen) was applied in the Jerusalem laboratory. "Spoligotyping" stands for "spacer-oligos." The process is based on PCR amplification of a series of 43 nonrepetitive short spacer sequences (of between 36 and 41 bp), located between small repeats (DRs) in the DR locus of the *Mycobacterium* genome [39]. This system differentiates between modern *M. bovis* and *M. tuberculosis,* as demonstrated by the array of the oligonucleotide sequences attached to a membrane [15]. The spacers are arranged on the blot in the order in which they appear in the H_{37}Rv DR sequence (spacers 1–19, 22–32, and 37–43) and the sequence of *M. bovis* BCG (spacers 20, 21, and 33–36). Subsequent sequencing of the DR regions of other *M. tuberculosis, M. bovis, M. microti,* and *Mycobacterium canetti* isolates has elucidated 65 novel spacer sequences (26 from *M. canetti*). The order of spacers in all isolates is very well conserved, apart from a few duplications of spacers [5, 6, 40]. One primer is biotinylated, and the amplified products are hybridized under stringent conditions to the membrane furnished in the Isogen kit. The final readout is made exquisitely sensitive by the binding of streptavidin–horseradish peroxidase to the biotinylated hybridized spacers, followed by use of a chemiluminescent detection system (Amersham).

Similar spoligotyping results for different DNA extractions can imply an accurate result based on the spacer regions of the genome or the spacer regions of a consistent pattern of fragmentation, perhaps because the molecule's stability varies at different sites along the DNA strand [38]. The applicability of this technique to ancient specimens has been established by Taylor et al. [38].

RESULTS

DNA recovery and sequencing. PCR amplification enabled recovery of *M. tuberculosis* complex at both laboratories. Sequencing revealed that the PCR product that was obtained belonged to a species in the *M. tuberculosis* complex. With use of primers for the S12 ribosomal gene, the isolated gel bands yielded a perfect match for a 163-bp fragment of the *M. tuberculosis* complex (of a possible 204 bp). IS6110 sequencing revealed a base-pair substitution difference. The London laboratory 92-bp segment had a 12-bp section missing (822–832 of X17348) in the forward strand, but the flanking sequences of 35 bp and 31 bp were identical to those in the database. A sequence for the reverse strand was obtained from bases 782–853, including the missing region on the positive strand. The total length sequenced was 92 bp, which included the total length between primers IS3 and IS4. Base discrepancy was specifically noted in the Jerusalem laboratory at 798 (T for C). However, the sequence obtained in London agreed with that in the database.

Extraction control samples and PCR control samples at both laboratories yielded no tuberculosis DNA. For all the other animal bone samples and specimens from the affected bone at locations away from the lesions, multiple extractions and PCR sequencing analyses found no evidence of tuberculosis DNA.

Spoligotyping. Spoligotyping revealed that the Pleistocene bison lesions contained *M. tuberculosis* complex segments not associated with modern *M. bovis* or *M. microti* [37]. The presence of spacers 39, 40, 41, and 43 eliminates the possibility that the DNA is from modern *M. bovis* [9, 10] or from the related BCG [9, 10], and thus eliminates the possibility that the sample was contaminated by DNA from a BCG-vaccinated individual. On the basis of identification of the spoligotype pattern as a 43-digit binary number, the pattern can be assigned descriptive nomenclature: taking a negative as 0 and positive as 1 and dividing the pattern into 6 blocks of 7 or 8 digits results in 6 blocks containing spacers 1–7, 8–14, 15–21, 22–28, 29–36, and 37–43. The binary number in each block can then be converted to the hexidecimal base. The result is a code of 6×2 digits [41]. The "hex code" for the bison pattern is thus 7F-6E-7E-7F-F8-7D.

The bison pattern was compared with a combined database of patterns collated by the National Institute of Public Health and Environment (RIVM), Utrecht, The Netherlands, and the Veterinary Sciences Division, Department of Agriculture and Rural Development, Belfast. The pattern did not exactly match any pattern in the database. Computer analysis use of BioNumerics 1.5 software (Applied Maths) was carried out to cal-

culate similarities by means of the Dice coefficient. The bison pattern was found to be most similar to pattern ST203 (hex code 7F-7F-7E-7F-F0-7F), with a similarity value of 93.2%. This pattern has been obtained from 2 *M. tuberculosis* isolates.

The pattern from the bison sample was then compared with a library of species-defined units. The highest average similarity to the bison pattern was obtained from the *M. africanum* unit (82.3%), followed by *M. tuberculosis* (76.6%) and *M. bovis* (72.7%; table 1). Therefore, the bison pattern fits well within the *M. africanum* and *M. tuberculosis* units. Further study of the bison spoligotype pattern by discriminant analysis shows that the bison pattern plots more closely to the *M. tuberculosis* group than to the *M. bovis* and *M. africanum* groups (figure 1). Principal-components analysis of the spoligotype patterns in the database, excluding those spacers that were negative for the bison pattern, shows that the bison pattern continues to group with *M. tuberculosis* and *M. africanum* rather than with *M. bovis* (figure 2). This demonstrates that grouping the bison pattern with *M. tuberculosis* and *M. africanum* is not related to any difficulties in obtaining a full spoligotype pattern.

DISCUSSION

Clarification of the evolutionary history of infectious diseases is advanced by comparison of DNA in ancient and modern specimens. Our understanding of the antiquity of tuberculosis was previously based upon presumptive, macroscopic pathology–based diagnosis of skeletal remains [16–30]. The work of Allison et al. [19], Cockburn et al. [42], Garcia-Frias [43], and Zimmerman [20] allowed histologic confirmation of *Mycobacterium*-related infection [18].

In the current study, DNA analysis of a 17,000-year-old skeletal specimen from Natural Trap Cave confirms the diagnosis of *M. tuberculosis* complex infection. IS6110 DNA fingerprinting is now the standard method for molecular analysis of tuberculosis epidemiology, because this sequence is apparently restricted to species that belong to the *M. tuberculosis* complex

Table 1. Average similarity of bison spoligotype pattern to a library of spoligotype patterns, divided into units based on species.

Pattern, by species of *Mycobacterium*	Average similarity to pattern from bison, %	Confidence factor[a]
M. africanum	82.3	1.33
M. tuberculosis	76.6	1.01
M. bovis	72.7	2.22
M. microti	17.4	20.31

[a] Calculated by comparing the average similarity between the bison pattern and the library unit's entries and the average similarities of the library unit's entries with each other, thus taking account of the heterogeneity of the library units.

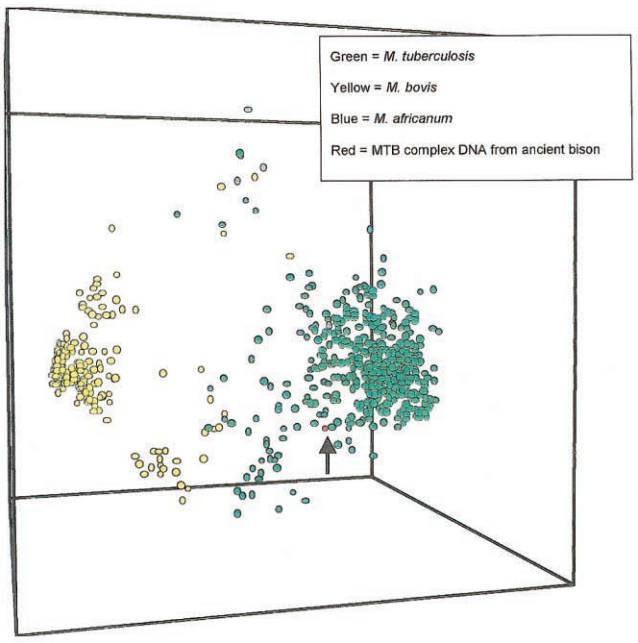

Figure 1. Pseudo–3-dimensional representation of discriminant analysis of 634 *Mycobacterium bovis, Mycobacterium tuberculosis,* and *Mycobacterium africanum* spoligotype patterns from a combined database of modern patterns collated by the National Institute of Public Health and Environment (RIVM), Utrecht, The Netherlands, and the Veterinary Sciences Division, Belfast. MTB, *M. tuberculosis* complex.

[39]. The results of our study further specified the particular species of this 4-member complex that is responsible for disease in bison.

M. bovis *and relationships.* It is clear from the presence of spacer elements 39, 40, 41, and 43 that the bison DNA is not modern *M. bovis* DNA. However, *M. bovis, M. tuberculosis, M. africanum,* and *M. microti* probably evolved from an *M. tuberculosis* precursor ~15,000–20,000 years BP [44], so the bison sample may be representative of the precursor or of group 1 species shortly after speciation.

Information from the *M. tuberculosis* genome sequence has very recently been used to construct a DNA microarray, in which almost every open-reading frame is displayed, allowing a global analysis of genetic differences between *M. tuberculosis, M. bovis,* and BCG substrains. Multilocus sequencing [44] had implied that there was a high degree of genetic conservation between *M. bovis* and *M. tuberculosis.* The new findings suggest that genetic diversity within the *M. tuberculosis* complex may be much greater than previously supposed and that gene deletion, rather than point mutation, may be a key source of genetic variation [9, 10].

M. microti. Van Soolingen et al. [37] demonstrated that *M. microti* could be recognized and distinguished on the basis of IS6110 restriction fragment–length polymorphism patterns and by means of spoligotyping. Although the former technique

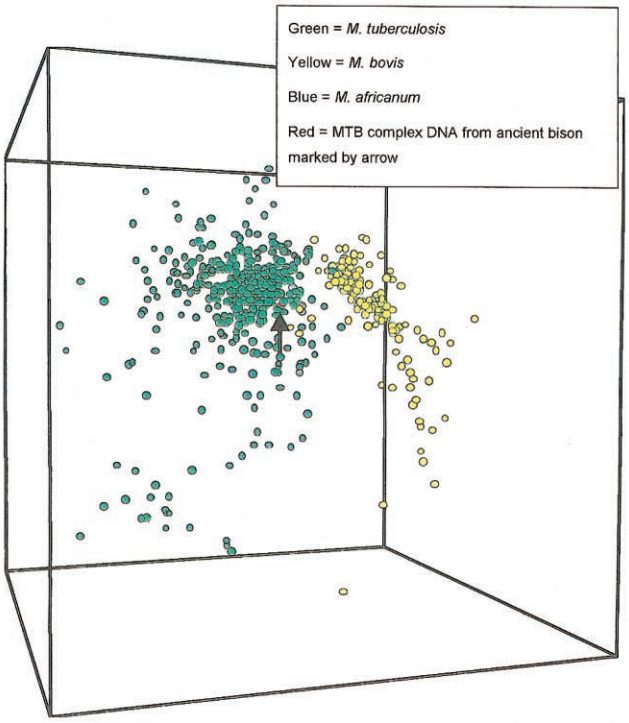

Figure 2. Pseudo–3-dimensional representation of principle-components analysis of 634 *Mycobacterium bovis, Mycobacterium tuberculosis,* and *Mycobacterium africanum* spoligotype patterns from a combined database of modern patterns collated by the National Institute of Public Health and Environment (RIVM), Utrecht, The Netherlands, and the Veterinary Sciences Division, Belfast. Analysis excludes spacers absent from spoligotyping of DNA from ancient bison (i.e., spacers 8, 10, 25–37, and 43). MTB, *M. tuberculosis* complex.

may be of limited value for application to ancient DNA because of taphonomic changes, the short direct-repeat regions of *M. microti* identified by spoligotyping allow its recognition and elimination as a causative organism for tuberculosis in Pleistocene bison. Although *M. microti* is not known to affect bovids, it does affect mice. Isolation of *M. tuberculosis* complex DNA from the lesion only and not from unaffected species and bones suggests that contamination of the site by mice is unlikely. Results of spoligotyping totally eliminate the possibility of such contamination.

M. africanum. *M. africanum* was originally described by Castets et al. [45]. It is responsible for 60% of cases of tuberculosis in Africa. Hass and des Prez [34] refer to *M. africanum* as intermediate between *M. tuberculosis* and *M. bovis*. Spoligotyping data support this. The possibly close relationship between the isolated organism and *M. africanum* is therefore perhaps not surprising. If the oldest identified organism (that described in this study) is more closely related to *M. africanum* than to other members of the *M. tuberculosis* complex, it would seem reasonable to consider this organism to be the primordial form of the organisms today recognized as *M. tuberculosis* and *M. bovis*.

M. tuberculosis *complex.* The Eisenach/Taylor primers are specific for the *M. tuberculosis* complex. Larger IS6110 primers and those for S12 are not. Hence, any product with the nested PCR is specific, as further documented by sequencing studies.

It is intriguing that the spoligotyping pattern of the bison isolate (as determined by comparison with the extensive collection of spoligotyping patterns in data banks) is closer to the pattern of modern *M. africanum* and *M. tuberculosis* than to the patterns of the other members of the *M. tuberculosis* complex. The patterns were compared to a database containing several hundred different patterns obtained from *M. tuberculosis* isolates, >170 *M. bovis* patterns, 34 *M. microti* patterns, and 6 *M. africanum* patterns. The pattern obtained from the bison material most closely matches with an *M. tuberculosis* pattern (figure 2). However, the bison pattern fits most closely with the mean of the *M. africanum* patterns as a group. The bison isolate, however, cannot be assigned to a particular species of the *M. tuberculosis* complex on the basis of spoligotyping data. Assignment of this isolate to any one of the species in the *M. tuberculosis* complex must be done with reservations, because speciation of the *M. tuberculosis* complex may not yet have occurred by 17,000 years BP. The *M. tuberculosis* complex is very homogeneous at the DNA level and may have speciated from a progenitor organism only very recently [44].

Caveats. The slight base-pair difference found at one laboratory between the ancient DNA from the bison specimen and modern sequences requires confirmation. It should be noted that there have been no reports of base changes in the 92-bp sequence of IS6110, although other tested samples have been considerably less ancient.

Base-pair differences do, however, provide an additional proof that contamination is not a factor. Kolman and Tuross [7] have criticized previous work because the investigators found ancient DNA that had sequences identical to those of modern DNA. They state that "the finding of identical sequences in an archaeological specimen and the control DNA sample precludes convincing proof that ancient DNA was extracted and analyzed" [7, p. 16]. The lack of a perfect match between the DNA extracted from the ancient bison and the DNA from contemporary organisms not only documents absence of contamination, but the character of that DNA suggests that it may represent the primordial organisms that developed into the organisms that are today used as positive controls.

CONCLUSION

This is the first DNA-based documentation of a macroscopically recognized tuberculosis infection in the fossil record. The conditions of Natural Trap Cave favored DNA preservation, be-

cause the temperature has remained nearly constant and relatively low, and the climate is semiarid with little water circulation. *M. tuberculosis* complex bacteria have hydrophobic cell walls, rich in long-chain mycolic acids. Location within bones provides further protection of DNA from environmental taphonomic loss [46].

Because this documentation is of tuberculosis occurring in the Pleistocene long before domestication of cattle, it suggests a different scenario for the spread of tuberculosis than is usually conceived [4, 21]. The presence of characteristic lesions in sheep *(Ovis canadensis catclawensis)* and extinct musk ox *(Bootherium bombifrons)* [31] and in bison—now confirmed as tubercular in origin—indicates that the disease was widespread throughout the Pleistocene. It is suggested that tuberculosis in bovids had a Holarctic pattern and that it reached North America at least 20,000 years BP. Absence of tubercular lesions in pronghorn *Antilocapra americanum* and *Equus* species suggests that it was absent in North America before the immigration of the advanced northern bovids.

Analysis of additional specimens that have tuberculosis lesions is now indicated to elucidate the evolution of tuberculosis. It is likely that the bison isolate contains some "novel" spoligotyping spacers—and perhaps spacers as yet not identified—between the spacers that appeared on the blot for our specimen. The paleontologic record provides an excellent geographic and chronological database for understanding disease origins and spread, especially for diseases with significant osseous impact.

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