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The diagnostic activity on wild animals through the description of a model case report (caseous lymphadenitis by *Corynebacterium pseudotuberculosis* associated with *Pasteurella* spp and parasites infection in an alpine ibex – *Capra ibex*)

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Abstract

The diagnostic activity on wild animals implies a hard laboratory protocol where multiple disciplines, from biology to pathological anatomy until the biotechnological techniques, must be integrated to establish the causes of death. To demonstrate these concepts, the analytical approach adopted for an alpine ibex (*Capra ibex*) found dead in a mountain area of North Western Italy was described. The animal showed sub-costal and lymph nodes collections of green-yellow pus, fibrinous pleuropneumonia and catarrhal-hemorrhagic enteritis. Purulent process was ascribed to *Corynebacterium pseudotuberculosis*, the causative agent of caseous lymphadenitis or pseudotuberculosis, pleuropneumonia to *Pasteurella multocida* and *Mannheimia haemolytica* and enteritis to *Mannheimia haemolytica*. Parasitic bronchopneumonia in the caudal lobes of the lung, a severe enteric infestation by gastro-intestinal and pulmonary strongyles and coccidia were found. The cause of death in the studied ibex appeared to be a consequence of an association between various pathological processes, with bacteriological and parasitic etiology.

Keywords: *Capra ibex*, Caseous lymphadenitis, *Corynebacterium pseudotuberculosis*, *Mannheimia haemolytica*, *Pasteurella multocida*.

Introduction

The diagnostic activity on wild animals involves application of hard protocol where multiple disciplines, from biology to pathological anatomy until the most advanced biotechnological techniques, must be integrated to achieve the final goal, in other words to establish the causes of death. The plural word “causes” is not a spelling mistake but a real affirmation, as it would be the case for free-living animals found dead in the territory, which - as is obvious - do not offer any clinical or anamnestic clues to the pathologist.

The infectious agents discussed in this case report (*Corynebacterium pseudotuberculosis*, Pasteurellaceae and different parasites) are generally detected in domestic animals with quite mild lesions limited to a single organ or system and so do not affect usually the survival of the animal. For example, in sheep and goat, *Corynebacterium pseudotuberculosis*, the etiological agent of caseous lymphadenitis (a worldwide spread wasting infectious disease), causes chronic purulent collections in different lymph nodes (especially subcutaneous) or internal organs, well confined by

capsule and “onion ring” structure (Dorella *et al.*, 2006; Baird and Fontaine, 2007; Fontaine and Baird, 2008).

Pasteurella spp. infections in cattle are generally localized in the low respiratory tract with consequent bronchopneumonia (Griffin, 2010). In the same way, the detrimental activity of parasites in domestic ruminants, as gastro-intestinal nematodes, are usually mild (diarrhea, loss of weight) and, however, can be avoided by the veterinary treatments and efficient control program (Stromberg and Gasbarre, 2006).

Nevertheless, in wild animals, living in an uncontrolled environment and exposed to many different stress factors, the outcome of these or other diseases frequently is severe or sometimes fatal. Therefore, in order to clarify these concepts, and following many years of practice in necropsy and lab diagnostics on wildlife, with analytical approach followed by explaining the pathological lesions showed in this report, a reference case of caseous lymphadenitis by *Corynebacterium pseudotuberculosis* associated with *Pasteurella* spp and parasites infection in an alpine ibex (*Capra ibex*) was proposed.

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Case Details

A carcass of an alpine wild ungulate, found by local rangers in the Aosta Valley region was brought to the lab for evaluating the possible causes of death.

After the collection of the data relating to the discovery area and the identification of the animal (species, sex, weight and age) by the reference biometric techniques, necropsy was performed. Organs and lesions, on the basis of the pathological features, were submitted to bacteriological examination (blood agar and McConkey agar with incubation at 37°C for 48 hours aerobically and anaerobically).

Exclusively for the intestine, a further specific bacteriological examination for *Campylobacter* spp (CCDA agar in microaerophilic condition for 24 hours at 42°C) and *Yersinia* spp. (CIN agar in aerobic condition for 24 h at 30°C) was applied.

The isolates were identified by Gram staining, biochemical galleries API (Biomerieux) and sequencing of the 16S rRNA. In addition to these analysis, a Real Time PCR (RT-PCR) technique was performed on conjunctival swabs for the detection of *Mycoplasma conjunctivae*.

The antibiotic sensitivity of the pathogenic isolates were tested by Kirby-Bauer routine method. Portions of organs, with or without gross lesions, were fixed in 10% buffered formalin and subsequently submitted to histological examination using haematoxylin and eosin staining method.

On the basis of the microscopic features, the Congo Red staining for the amyloid substance, with and without a pre-treatment by a solution of potassium permanganate and sulphuric acid, was also performed. Parasitological examination for the intestinal content was carried out using a 100% zinc sulphate solution.

Discovery area

The carcass of the animal was found at the end of October 2015 in Aosta Valley region (North Western Italy) nearby Peson, a resort in the municipal territory of Oyace, at an altitude of 1300 m.

Animal identification

The animal has been identified as a male ibex (*Capra ibex*). The age, based on the growth of the horns (Fig. 1), was estimated to be about four years and half. The weight was equal to 33 Kg.

Gross lesions

The carcass of the animal showed moderate degenerative changes and discrete emaciation; extensive left sub-costal purulent collection, characterized by granular-creamy texture and yellow-green colour exudate, with compression and partial collapse of the left lung (Fig. 2).



Fig. 1. *Capra ibex* - Age estimation based on the growth rings of the horns.



Fig. 2. *Capra ibex* - Sub-costal purulent collection associated with pleural effusion.

Similar purulent collections in various lymph node groups (in particular subcutaneous and mediastinal), fibrinous pleuropneumonia characterized by bilateral consolidation of lung apical lobes with dark red colour and interlobular oedema (Fig. 3), caudal lung lobes with focal green-grey areas of hepatization and catarrhal-hemorrhagic enteritis.



Fig. 3. *Capra ibex* - Consolidation and interlobular oedema of the lung cranial lobe adjacent to the purulent collection.

Bacteriology, antibiotic sensitivity test and RT-PCR for *Mycoplasma conjunctivae*

Sub-costal and lymph nodes abscess: on blood agar, growth of chalky and rough pinpoint colonies at 24 hours of incubation, more apparent at 48-72 hours and surrounded by total haemolysis haloes. Gram positive coccobacilli were observed (Fig. 4) that identified by biochemical tests as *Corynebacterium pseudotuberculosis* (negative for nitrate reductase).

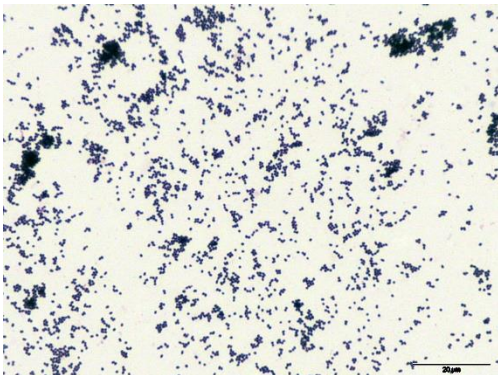


Fig. 4. *Capra ibex* - *Corynebacterium pseudotuberculosis* isolated from the lesion shown in Figure 2 (Gram staining).

For antibiotic sensitivity, and referring to M45 3rd ed. 2016 CLSI guideline, the isolates were sensitive to amoxicillin, amoxicillin and clavulanic acid, ampicillin, cephalothin, cefoperazone, chloramphenicol, enrofloxacin, gentamicin, oxytetracycline, penicillin, tetracycline, thiamphenicol, erythromycin, bacitracin, lincomycin and sulfamethoxazole/trimethoprim.

Lung: on blood agar, isolation of two types of colonies, colony A was mucoid and dew drop like, and colony B was regular round, smooth, greyish, surrounded by a faint halo of partial haemolysis. Also characterized by stunted growth and pinpoint colonies on McConkey agar. Both colonies were Gram-negative coccobacilli, occasionally filamentous, with more or less evident capsule (Fig. 5).

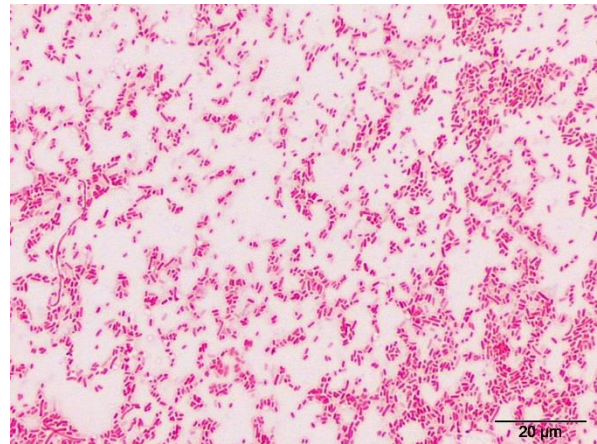


Fig. 5. *Capra ibex* - *Mannheimia haemolytica* isolated from the lesion shown in Figure 3 (Gram staining).

Both strains were attributed morphologically and by biochemical tests to the species *Pasteurella* and later identified by 16S rRNA sequencing as *Pasteurella multocida* (colony A) and *Mannheimia haemolytica* (colony B).

For antibiotic sensitivity, and referring to VET01S 3rd ed. 2015 CLSI guideline, *Pasteurella multocida* was sensitive to amoxicillin, amoxicillin and clavulanic acid, ampicillin, cephalothin, cefoperazone, chloramphenicol, enrofloxacin, gentamicin, oxytetracycline, penicillin, tetracycline, thiamphenicol and sulfamethoxazole/trimethoprim; intermediate to erythromycin; and resistant to bacitracin and lincomycin.

Whereas *Mannheimia haemolytica* was sensitive to amoxicillin, amoxicillin and clavulanic acid, ampicillin, cephalothin, cefoperazone, chloramphenicol, enrofloxacin, gentamicin, lincomycin, oxytetracycline, penicillin, sulfamethoxazole/trimethoprim, tetracycline and thiamphenicol; intermediate to erythromycin; and resistant to bacitracin.

Intestine: on blood agar, isolation of same colony B observed in the lung and identified as *Mannheimia haemolytica*, with absence of *Campylobacter* spp. and *Yersinia* spp.

Liver, bone and brain: absence of pathogenic bacteria.

The RT-PCR for *Mycoplasma conjunctivae* from eye swabs was negative.

Microscopic lesions

Sub-costal abscess: inflammatory areas, with colliquative necrosis in the central parts, coagulative necrosis and occasional foci of calcification more in the peripheral areas; these areas were surrounded by spongy macrophages mixed with lymphocytes and, more outside, by mature fibroblasts and fibrous connective tissue, with rich neovascularization. In the intercostal muscles, multiple cysts of sarcosporidia (*Sarcocystis* spp.) were present (Fig. 6).

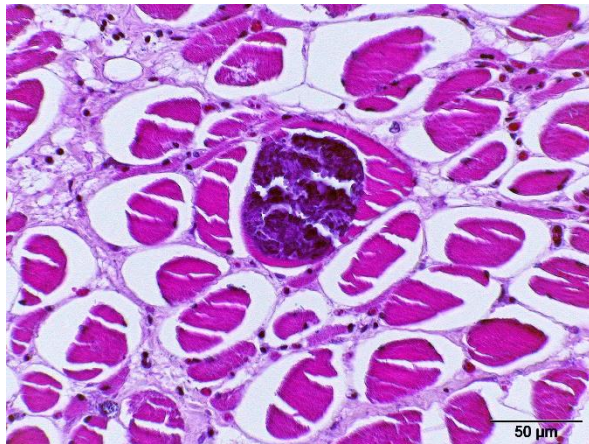


Fig. 6. *Capra ibex* - *Sarcocystis* spp. inside an intercostal muscle fiber (Haematoxylin and eosin staining).

Abscess of lymph nodes: same aspects observed in sub-costal lesion (except the parasitic cysts).

Lung: blood congestion associated with hemorrhages and interstitial monocytes were observed in the apical lobes. In the caudal lobes, areas of interstitial pneumonia, characterized by the presence of nematode eggs and larvae (Fig. 7), hypertrophy of smooth muscle septa, hyperplasia of the bronchial-associated lymphoid tissue (BALT), emphysematous areas with dilation and rupture of alveolar walls.

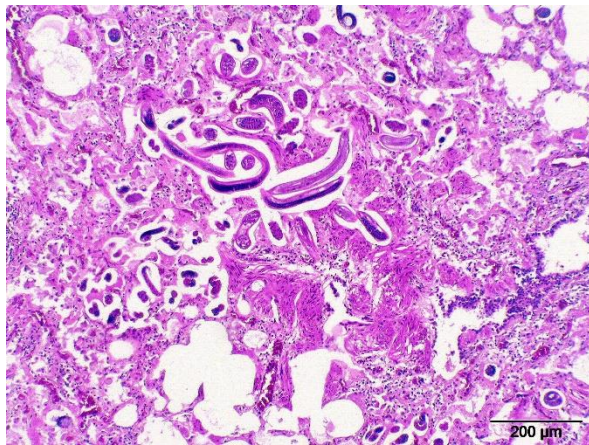


Fig. 7. *Capra ibex* - Nematodes larvae inside an area of the lung (Haematoxylin and eosin staining).

Liver: extensive deposits of amorphous, eosinophilic and homogeneous material were found between the hepatocytes and in the portal spaces (Fig. 8), mostly arranged in round aggregates with fibrillar border, identified as amyloid AA (secondary type) on the basis of the Congo Red affinity and the typical birefringence of apple green colour at polarized light observation, a feature that disappears after treatment of the sections with potassium permanganate-sulphuric acid solution.

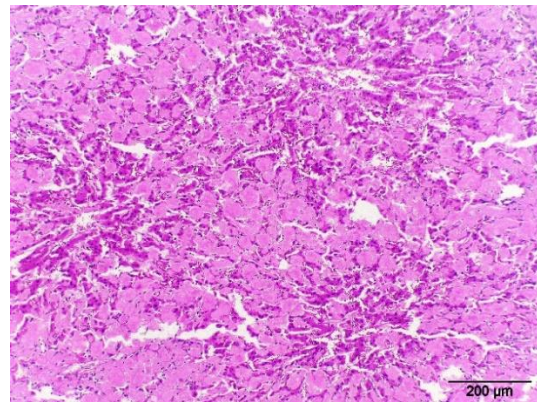


Fig. 8. *Capra ibex* - Deposits of amyloid between the hepatocytes (Haematoxylin and eosin staining).

Kidney: despite the partial autolysis of tissues, the same aspects seen in the liver were observed, with deposits of amyloid inside the glomerular mesangium (Fig. 9) and in the intertubular spaces of renal medulla.

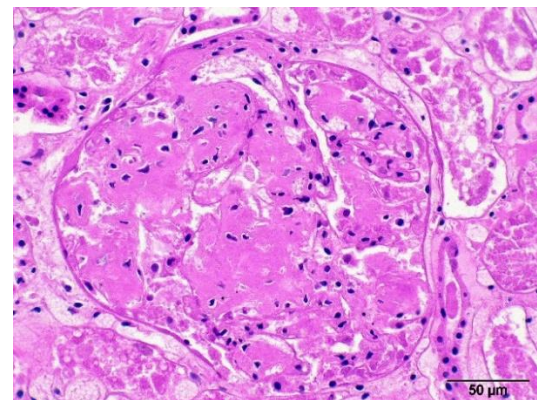


Fig. 9. *Capra ibex* - Deposit of amyloid in a renal glomerulus (Haematoxylin and eosin staining).

Intestine: despite the severe autolysis of tissues, extensive amyloid deposits were detected in the lamina propria.

Eye: absence of histological lesions.

Parasitological examination of faeces

High level of infestation with the presence of intestinal strongyles eggs (with a prevalence of *Nematodirus* spp.), larvae and eggs of pulmonary strongyles and coccidia oocysts (Fig. 10).

Discussion

The carcass weight, based on the growth curves available in the literature - even if referred to the Swiss ibex (Mustoni *et al.*, 2002) - and taking into account the physiological loss in the winter, was below the normal (which should correspond to about 45 Kg); also with the physical examination, the carcass was rather emaciated, with regression of the major muscle groups (especially the gluteal muscles).

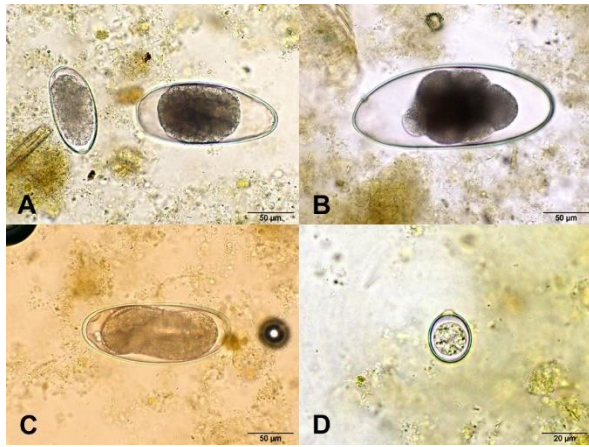


Fig. 10. *Capra ibex* - Eggs of parasites observed in the intestinal content. (A): gastro-intestinal strongyles; (B): *Nematodirus* spp.; (C): pulmonary strongyles; (D): coccidia.

The main gross lesions, in other words the purulent sub-costal and lymph nodes collections and pulmonary hepatization areas, were ascribed respectively to the intervention of *Corynebacterium pseudotuberculosis* and *Pasteurella* spp in combination with parasites. *Corynebacterium pseudotuberculosis* is the causative agent of caseous lymphadenitis or pseudotuberculosis, an infectious disease that is characterized by the onset of multiple abscesses, mostly at the level of subcutaneous lymph nodes and internal organs, with typical yellow-green pus.

The infection, considered a minor zoonosis especially for those involved in shearing and slaughtering of target animals, can affect domestic (mostly sheep and goats) and wild ruminants (Dorella et al., 2006; Baird and Fontaine, 2007); among the latter, besides the alpine ibex, cases have been reported in spanish ibex (*Capra pyrenaica*) (Cadena-Colom et al., 2014), alpine chamois (*Rupicapra r. rupicapra*) (Bassano et al., 1993), fallow deer (*Dama dama*) (Pérez et al., 1996), red deer (*Cervus elaphus*) (Matos et al., 2015), tailed deer (*Odocoileus virginianus*) (Stauber et al., 1973), pronghorn (*Antilocapra americana*) (Clark et al., 1972), white-tailed gnu (*Connochaetes gnou*) (Müller et al., 2011), oryx (*Oryx leucorix*) (Tarello and Theneyan, 2008) as well sporadically and atypically in not-artiodactyls animals such as the cheetah (*Acinonyx jubatus*) (Boomker and Henton, 1980) and the orycteropus (*Orycteropus afer*) (Roth and Vickers, 1966).

Two subtypes of *Corynebacterium pseudotuberculosis* are classically recognized, biovar *equi* (mostly isolated from bovine and horse) and biovar *ovis* (mostly isolated from goat and sheep), respectively differentiated for their ability to reduce nitrates or not.

The main virulence factor of *Corynebacterium pseudotuberculosis* is phospholipase D, an enzyme capable of destroying the cell membranes. Based on the

biochemical results, the strain involved in this case, resulting negative for nitrate reductase, can thus be classified as biovar *ovis*, consistent with the fact that precisely this type would be more widespread among small ruminants. The severity of purulent lesions, the remarkable extension and the high degree of liquefaction without the characteristic tendency to stratification (generally observable in domestic sheep and goats and described as “onion ring” appearance), supports the hypothesis that the strain isolated is particularly aggressive or that the immune status of the ibex was particularly depressed and therefore not able to adequately counteract the progression of infection. The general histological features, namely colliquative and coagulative necrosis, absence of caseous necrosis, type of phlogosis and mineralized foci were described before for caseous lymphadenitis in small ruminants (Baird and Fontaine, 2007).

As regards the antibiotic-resistance, the strain was sensitive to all classes of antimicrobials, in particular beta-lactam antibiotics which are the molecules of greater use for the treatment of pseudotuberculosis in domestic animals.

In the lungs, two species of Pasteurellaceae were isolated; *Pasteurella multocida* and *Mannheimia haemolytica*, that reported by numerous authors and frequently found in wild ungulates (Richard et al., 1992; González-Candela et al., 2006; Richomme et al., 2006). Based on our experience (personal unpublished data), acquired mainly on the pneumonia of wild alpine ruminants (chamois and secondly roe deer, ibex and deer), *Mannheimia haemolytica* is more involved. Out of 74 lung affections, with lesions characteristic of Pasteurellaceae (in particular fibrinous pleuropneumonia in apical lobes associated to pleural exudation); *M. haemolytica* was the causative agent in 51 cases, and *P. multocida* in 23 cases. Strains of *Mannheimia*, generally identified with biochemical methods, it is likely, on the basis of what is reported by some researchers (Villard et al., 2006; Posautz et al., 2014), that a certain proportion may correspond to the T biotype, recently reclassified as *Pasteurella trehalosi*. Pasteurellosis, both for domestic and wild ruminants, can be fatal in severe or complicated cases, when the lesions involve more of the lung tissue, over the classical localization in apical lobes.

In our case, *Mannheimia haemolytica* was isolated also from, atypical site, the intestine, which may suggest a septicemic event. The strain seems to be responsible of the observed enteritis, considering the absence of intestinal pathogens such as *Salmonella* spp., *Yersinia* spp. and *Campylobacter* spp., and the presence of amyloid deposit.

In light of the good sensitivity to many antibiotics of choice, the isolated strains of *Pasteurella* maybe did not come from domestic livestock.

Pulmonary disease appeared further aggravated by the presence of eggs and larvae of nematodes, especially localized in the caudal lobe. In other studies on the ibex, there was a prevalence of *Muellerius* spp. and *Protostrongylus* spp., with different findings of *Cystocaulus ocreatus* and *Dictyocaulus filaria* in *Capra ibex* and *Capra pyrenaica* (Alasaad et al., 2009; Cassini et al., 2015).

In addition to these classic microscopic findings related to different etiologic agents involved, it is interesting to consider the severe and pervasive presence of secondary amyloid (type AA) in the liver, kidney and intestine. The secondary systemic amyloidosis, also called acquired amyloidosis, corresponds to the deposit - in the tissue extracellular spaces - of serum acute phase proteins (SAA) as a result of an overstimulation of the immune system caused by chronic diseases (such as tuberculosis or rheumatoid forms), stress, trauma and tumors. Among the wild ungulates, a similar form was reported, with renal medullary amyloidosis, in the gazelle (Rideout et al., 1989). In the ibex, a direct relationship between the increase of acute phase proteins and the progressive severity of the mange by *Sarcoptes scabiei* was reported (Ráez-Bravo et al., 2015).

In our case, the systemic amyloidosis is probably connected to the general pathological condition, maybe especially to the intervention of *Corynebacterium pseudotuberculosis* which causes, as already described, a chronic debilitating disease with purulent collections that spread by tissue contiguity or lymphatic vessels. The hepato-renal failure, evidenced by severe involvement of liver lobules and most kidney glomeruli, has probably caused the death of the animal. Considering the serious outbreaks of keratoconjunctivitis by *Mycoplasma conjunctivae* among ibex and chamois of Western Alps in the past years (Giacometti et al., 2002), in our case, the animal was not affected by *Mycoplasma conjunctivae* as there was no eye affections and confirmed by the negative result of RT-PCR.

As mentioned in the introduction, the routine diagnostics on alpine wild ungulates found dead on the territory shows that the decrease of an animal free-living, in most of that cases where the traumatic events are however excluded, depends on an unfortunate association between different pathological processes. It's known that wild animals have to face up a large number of stressors, in particular adverse environmental conditions such as extreme temperature, shortages of pasture or pressure of predators; these factors, when are more aggressive, probably depress the immune status of the animal, promote the progression of microorganisms in the host tissues and emphasize the pathogenic action of infectious agents, even those generally mild in livestock.

More than any other, the case report described above demonstrates these conclusions, considering that a relatively young ibex died for the co-infection of five different pathogens (bacteria, protozoan and metazoan parasites). In our opinion, primary pathogenic infection was by *Corynebacterium pseudotuberculosis* (which caused a severe sub-costal purulent collection with the collapse of the corresponding lung and - as we have yet observed in other cases of pseudotuberculosis - a generalized secondary amyloidosis), followed by complications with *Mannheimia haemolytica* (found in a septic form with the involvement of lung and intestine), *Pasteurella multocida*, nematodes and coccidia.

Conflict of interest

The authors declare that there is no conflict of interest.

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