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Review Article

A review of infection of wildlife hosts with *Mycobacterium bovis* and the diagnostic difficulties of the 'no visible lesion' presentation

D Gavier-Widén[*§], MM Cooke[†], J Gallagher[‡], MA Chambers[#] and C Gortázar[¥]

Abstract

The pathology, frequency and diagnostic implications of 'no visible lesion' (NVL) tuberculosis (Tb), i.e. infection with *Mycobacterium bovis* in the absence of macroscopic lesions, are described in a wide taxonomic range of wildlife hosts. Information collected and evaluated on the definition and occurrence of NVL Tb, histopathological characteristics, post-mortem techniques to detect minimal lesions, and diagnostic difficulties revealed most Tb-infected individuals with NVL had minute tuberculous lesions, which were difficult to see by eye. Acid-fast organisms (AFO) were sometimes detected in the lesions. Ideally, mycobacterial culture of pools of lymph nodes and/or oropharyngeal tonsils is necessary for the accurate diagnosis of Tb in the absence of macroscopic lesions. At a very minimum, the diagnostic methods applied for studying the prevalence of Tb in the population should be clearly described, to allow comparison between studies.

KEY WORDS: ***Bovine tuberculosis, Mycobacterium bovis, wildlife, Eurasian badger (Meles meles), brushtail possum (Trichosurus vulpecula), cervids, ferrets (Mustela furo), European wild boar (Sus scrofa)***

Introduction

Bovine tuberculosis (Tb) is an important re-emerging zoonotic disease caused by infection with *Mycobacterium bovis* and is one of the most important diseases affecting free-ranging and farmed wild animals in many parts of the world. Implementation of eradication programmes for cattle have successfully eradicated the disease from some countries, including Australia, Switzerland, most European Union member countries, Canada and most states in the United States of America (USA) (de la Rua-Domenech *et al*. 2006). Similar programmes in other countries have reduced the incidence in the cattle population but sporadic outbreaks still occur, which have been attributed to the presence of a wildlife reservoir responsible for maintenance and spread of *M. bovis* in the wild. *Mycobacterium bovis* is able to infect a wide range of species, including members of the orders Marsupialia, Carnivora, Primates, Rodentia, Lagomorpha, Artiodactyla, and others (Francis 1958; Kovalev 1980). Some examples of wildlife reservoirs are brushtail possums (*Trichosurus vulpecula*) in New Zealand (Davidson 1976; O'Neil and Pharo 1995), Eurasian badgers (*Meles meles*) in Britain (Muirhead *et al*. 1974; Nolan and Wilesmith 1994), European wild boar (*Sus scrofa*) in some regions in Spain (Martín-Hernando *et al*. 2007; Gortazar *et al*. 2008; Naranjo *et al*. 2008), Kafue lechwe antelope (*Kobus leche kafuensis*) in Zambia (Clancey 1997), African buffalo (*Syncerus caffer*) in South Africa (Bengis *et al*. 1996) and Uganda (Renwick *et al*. 2007), white-tailed deer (*Odocoileus virginianus*) in Michigan, USA (O'Brien *et al*. 2006), and wood bison (*Bison bison athabascae*) and elk (*Cervus elaphus manitobensis*) in Canada (Nishi *et al*. 2006).

Tuberculosis in deer is of increasing concern. Deer in captivity and wild cervids of various species have been widely diagnosed as infected with *M. bovis* (Clifton-Hadley and Wilesmith 1991; O'Brien *et al*. 2004, 2006; Vicente *et al*. 2006). Importation of farmed deer was responsible for the re-introduction of Tb in areas or countries that had been free of the disease (Bölske *et al*. 1995). Wild deer may act as an important, or even the main, initiator of new areas of infection in wildlife and may behave as maintenance hosts (Schmitt *et al*. 1997; Lugton *et al*. 1998; Vicente *et al*. 2006).

Understanding the role of wildlife in the maintenance and transmission of *M. bovis* infection is essential for the design of eradication or control strategies, not only to protect livestock and people but also to find methods to protect the wild animal species involved (de Lisle *et al*. 2001). In ecosystems with multiple susceptible hosts, several factors are important in determining the epidemiological role of the species, for example the species composition of the ecosystem, the behaviour and abundance of the host species (de Lisle *et al*. 2001), their ecology and the characteristics of the tuberculous infection, including its rates of inter- and intra-species transmission (reviewed by Holt *et al*. 2003; Corner 2006; Delahay *et al*. 2007; Renwick *et al*. 2007). Certain species, depending on the combination of the multiple factors mentioned above, usually have a minor role in the persistence of the infection and are considered to be spillover hosts; these often contract Tb by scavenging or predating on infected animals. In spillover hosts species, infection with *M. bovis* occurs or persists only sporadically. Some examples include feral cats (*Felis catus*), stoats (*Mustela erminea*) (Ragg *et al*. 1995a), ferrets (*Mustela furo*) (Caley and Hone 2005), European hedgehogs (*Erinaceus europaeus*) (Lugton *et al*. 1995), rats (*Rattus norvegicus*) (Little *et al*. 1982), and coyotes (*Canis latrans*) (Bruning-Fann *et al*. 1998). If

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AFO Acid-fast organism(s)
NVL No visible lesion(s)
Tb Tuberculosis

these animals do not spread the infection they are referred to as dead-end hosts (de Lisle *et al*. 2001). When referring to one single species, the term maintenance host is synonymous with the term reservoir host. A maintenance or reservoir host species is able to maintain *M. bovis* infection in a locality by transmission between individuals of the same species in the absence of any other source of *M. bovis*. Maintenance or reservoir hosts are required for the infection to persist in an area (de Lisle *et al*. 2001). The number of species reported in the categories of reservoir and spillover hosts is large and the same species may be regarded as one or the other in different situations or contexts. For example, ferrets may be maintenance hosts in areas of high population density but spillover hosts elsewhere (Caley and Hone 2005). Also, while European wild boar are able to maintain Tb infection in the wild and are most probably able to transmit the disease to other species, thus acting as a true wildlife reservoir, at least in certain areas in Spain (Naranjo *et al*. 2008), in other conditions, in Australia and New Zealand, feral pigs are considered to be spillover hosts (Corner *et al*. 1981; McInerney *et al*. 1995). Any spillover host may become an important source of infection for other wildlife, and occasionally livestock (Ragg *et al*. 1995b). Spillover infection can also pose a threat to endangered species, exemplified by the Iberian lynx (*Lynx pardinus*) (Pérez *et al*. 2001), and vulnerable species such as the lion (*Panthera leo*) and cheetah (*Acinonyx jubatus*) (Michel *et al*. 2006).

The propensity to develop severe disease varies among species (Francis 1958). For example, carnivores are considered more resistant than cervids. Indeed, the development, severity, morphology and distribution of lesions show differences in the various hosts. For wildlife surveillance applications and diagnostic purposes it is important to understand how infection is manifested in the different species. A form of *M. bovis* infection easily overlooked is the 'no visible lesion' (NVL) presentation of Tb (Gallagher *et al*. 1998), i.e. infection in the absence of macroscopic lesions at post-mortem examination. The primary aim of this review is to describe and discuss NVL Tb in some wildlife hosts, to highlight the diagnostic difficulties in dead animals, and increase awareness of this presentation of the disease.

Definition of NVL Tb and its occurrence

Strictly speaking, the term NVL simply implies absence of macroscopic lesions of any origin. In this review, we used the designation NVL Tb to describe individuals with confirmed *M. bovis* infection by culture which showed no detectable macroscopic lesions at post-mortem examination. In general, thorough histopathological examination of individuals with NVL Tb revealed that most of them had tuberculous lesions, but which were so small they were not detectable during routine diagnostic necropsy. The term NVL Tb has also been applied in a different context, i.e. to cattle with positive tuberculin skin tests but no macroscopic tuberculous lesions. This is due in some cases to false-positive skin-test reactions because of sensitisation by *M. avium* complex or non-pathogenic mycobacteria. However, confirmed *M. bovis* infection in the absence of macroscopic lesions, e.g. true NVL Tb, has also been described in cattle, affecting 10.9% of positive skin-reactor animals and 30.4% of in-contact, clinically healthy cattle with negative skin tests selected from reactor herds (Liebana *et al*. 2008).

Historically, the concept of NVL Tb developed as the result of an observation in free-living Eurasian badgers. Tuberculosis due to *M. bovis* in this species in the British Isles was first recognised in 1971 (Muirhead *et al*. 1974); the gross morphological presentation of various stages or severity of Tb was elaborated. It was found that culture of a pool of lymph nodes obtained at post-mortem examination, comprising retropharyngeal, mesenteric and bronchomediastinal lymph nodes, revealed a significant proportion of badgers that had no macroscopic lesions were actually infected with *M. bovis* (Gallagher *et al*. 1976); those badgers were referred to as 'NVL'. The proportion of badgers with presentation of NVL Tb varied from 30% to approximately 80% of all infected badgers in certain areas of the United Kingdom (UK) and Ireland (Clifton-Hadley *et al*. 1993; Fagan 1993; Gallagher *et al*. 1998; Crawshaw *et al*. 2008).

No visible lesion Tb has also been studied in other species. In New Zealand, 27.8% of feral ferrets that were confirmed as infected showed no macroscopic lesions (Lugton *et al*. 1997). Further studies on ferrets showed that there was an association between the presence of macroscopic lesions and the numbers of cfu of *M. bovis* present in the pool of lymph nodes. Fewer cfu were cultured from ferrets with NVL Tb than from ferrets with macroscopic lesions, reflecting lower numbers of bacteria (de Lisle *et al*. 2005). Also in New Zealand, histopathological examination of an extensive range of tissues from 117 tuberculous brushtail possums revealed only eight (6.8%) with no macroscopic lesions (Cooke 2000a). Two single cases of *M. bovis* infection were reported in wild mink (*Mustela vison*) in South Wales and Ireland, neither of which had lesions detectable macroscopically (Delahay *et al*. 2002). In a survey in carnivores and omnivores in an area of endemic Tb in white-tailed deer in Michigan, *M. bovis* was cultured from pools of lymph nodes from six coyotes, two raccoons (*Procyon lotor*), one red fox (*Vulpes vulpes*) and one black bear (*Ursus americanus*). These animals had probably become infected by consuming tuberculous deer. Of all those infected animals only two of the coyotes had macroscopic lesions (Bruning-Fann *et al*. 1998, 2001). Similarly, a red fox in Spain infected with *M. bovis* (Martin-Atance *et al*. 2005) and the majority (28/29) of the infected red foxes in the UK examined between 1980 and 2007 (Gallagher 1980; Little *et al*. 1982; Delahay *et al*. 2007) showed no macroscopic lesions.

Several reports describe NVL Tb in deer. In captive elk, 7% of culture-positive animals showed no macroscopic lesions at inspection in an abattoir (Rohonczy *et al*. 1996). Lugton *et al*. (1998) reported that, in a comprehensive study of natural infection in red deer (*Cervus elaphus*), 28% of the *M. bovis*-infected animals showed no detectable macroscopic lesions of Tb. Higher proportions of NVL Tb in red deer were found in Spain. Studies conducted in 2006–2007 in Doñana National Park, an endemic Tb area with a high prevalence of the disease in wild ungulates, showed that 30% of infected red and fallow deer (*Dama dama*) had NVL Tb (C Gortázar, unpubl. obs.). In a fenced population with a lower prevalence of Tb, sampled from 2000 to 2007, 50% of all *M. bovis*-infected red deer had NVL Tb (C Gortázar, unpubl. obs.). Visible tuberculous lesions in deer may be missed if the inspection does not include the head, thoracic cavity and abdominal cavity (Vicente *et al*. 2006).

In European wild boar from endemic Tb areas in Spain, macroscopic inspection failed to detect 22/127 culture-positive cases of Tb; an NVL rate of 17% (Martín-Hernando *et al*. 2007). Interestingly, post-mortem detection of visible lung lesions was rare in wild boar (11%), but histopathology revealed that 38% of culture-positive animals actually had small (NVL) lung lesions.

Rodents in general have been considered relatively resistant to bovine Tb (Francis 1958; Coleman and Cooke 2001). However, a low prevalence of infection has been documented in a few individuals in the UK, including brown rats (*Rattus norvegicus*), the wood mouse (*Apodemus sylvaticus*), yellow-necked mouse (*Apodemus flavicollis*), and field vole (*Microtus agrestis*). No macroscopic lesions were detected in these species (Little *et al*. 1982; Delahay *et al*. 2002, 2007). In some wild animal species, such as moles (*Talpa europaea*), the occurrence of NVL Tb has not been studied on a large scale. Isolated data suggest that this presentation may be more frequent than previously recognised.

As suggested above, the prevalence of NVL Tb may be related to the species of the host. However, several other factors may also contribute to or affect the prevalence of NVL cases; for example the methods of examination or analyses used. These are discussed below.

Diagnostic methods

Post-mortem examination: NVL Tb and detection of minimal lesions

Diagnosis based on detection of macroscopic lesions followed by mycobacterial culture of only the lesions may have low sensitivity in identifying tuberculous animals if NVL Tb for the species is common. As a general principle, the detection rate will be a function both of the number of sites examined and how carefully each is examined. This was exemplified for the diagnosis of Tb in cattle (Corner *et al*. 1990) and badgers (Crawshaw *et al*. 2008).

Conversely, macroscopic lesions of Tb may be wrongly attributed to other diseases. For instance, in deer, tuberculous lesions may be confused with those of pneumonia due to other causes, e.g. actinobacillosis, parasitic hepatitis, tonsillar cysts, or other mycobacterioses such as Johne's disease (Rohonczy *et al*. 1996). Intercurrent disease may make tuberculous lesions difficult to visualise. In ferrets, frequent infection with adiaspores of the fungus *Chrysosporium parvum* var. *crescens* caused 1–2-mm palpable lesions in the pleura and parenchyma of the lung, which may have been so numerous that they resembled miliary pulmonary Tb (Lugton *et al*. 1997). In Eurasian badgers, besides adiaspiromycosis, small pulmonary lesions may be due to parasites (*Aelurostrongylus abstrusus* or *A. falciformis*), which are indistinguishable macroscopically from small tuberculous lesions (Gallagher *et al*. 1998). Similarly, adiaspiromycosis may be confused for Tb in possums (Cooke *et al*. 1995). In general, parasitic, fungal, bacterial and other lesions (e.g. lipid, foreign body) may confound the diagnosis of macroscopic tuberculous lesions in any species, and diagnosis should be confirmed by culture, and/or other methods.

Inspection of lymph nodes and oropharyngeal tonsils

The selection of lymph nodes to be inspected depends on the species, but in general includes the parotid, mandibular, medial retropharyngeal, mediastinal, bronchial, mesenteric, renal, hepatic and iliac lymph nodes, which represent most infections of organs. The European wild boar is an exception to this in that inspection of the mandibular lymph node is sufficient for the detection of >90% of cases of Tb (Martín-Hernando *et al*. 2007). However, tuberculous lesions may occur in several tissues that are not routinely inspected or sampled. For example, in the badger, lesions have been found in the seminal vesicles (Gallagher *et al*. 1976), salivary gland (Cooke 2000b), and small and large intestine (Gavier-Widén *et al*. 2001); and in possums, in the mammary glands and thymus (Jackson *et al*. 1995), and small and large intestine (Cooke 2000a).

Single lesions in lymph nodes are common in tuberculous badgers and ferrets. If not all of the commonly affected lymph nodes are inspected, single tuberculous lesions in carcasses could easily be overlooked. Lesions in lymph nodes may be single and small (<2 mm) without accompanying enlargement of the lymph node, and may be the only tuberculous lesion in the carcass. Slicing the complete lymph node as thinly as possible with a sharp scalpel significantly increased the chances of finding such lesions (Corner *et al*. 1990).

Tuberculosis may be contracted through fighting or bite wounds. In different studies, evidence of tuberculous bite wounds was identified in 13–32% of Eurasian badgers (Fagan 1993; Gallagher *et al*. 1998). Skin lesions may be difficult to detect in animals with thick hair or fur, and may be better visualised in superficial lymph nodes draining wounds. In possums, both macroscopic and microscopic lesions were recorded more often in the superficial lymph nodes than in the lower respiratory tract, and were marginally more common in males than females (Cooke 2000a). Agonistic interactions between animals of this species tend to occur more commonly between males than females (Day *et al*. 2000), and lesions in superficial lymph nodes may be due to lesions in the skin, inflicted during fighting. Due to their thick, dense fur, only markedly obvious skin lesions, such as torn ears, would be detected during post-mortem examinations of possums. Also, 14/17 possums with single macroscopic lesions had them located in superficial lymph nodes (Cooke 2000a). Thus, all the superficial lymph nodes should be inspected in badgers and possums.

The oropharyngeal tonsils are frequent sites of mycobacterial infection, often in the absence of macroscopic lesions, particularly in deer (Lugton *et al*. 1998; O'Brien *et al*. 2004). Therefore in this species, these tonsils should be inspected and included with pools of lymph nodes for culture. In deer, it is also important to carefully inspect and sample the mesenteric lymph nodes, and the ileocaecal valve and associated lymph nodes, since lesions are often found in this region (Vicente *et al*. 2006). However, lesions in these locations must be distinguished from those caused by other mycobacteria frequently found in deer, such as *M. avium* subsp. *paratuberculosis* (Johne's disease) (Balseiro *et al*. 2008; Reyes-García *et al*. 2008).

Inspection of the respiratory system

Finding small tuberculous lesions in major organs, such as the lungs, is often more difficult than in the lymph nodes because of the size of the organ, particularly in very large animals. The upper and lower respiratory tracts should be cut open and inspected, as tuberculous granulomas may occur at any site. Serial thin slicing (up to 5 mm thick) of each lung lobe, using a long sharp knife, followed by palpation of the slices, may be the only means of finding small pulmonary lesions. A second macroscopic inspection can be performed on formalin-fixed material. Studies on NVL tuberculous badgers showed that serial thin slicing of formalin-fixed lung, using a bacon slicer, was easier than of fresh tissues, as fixation made the tissues firmer (Gallagher *et al*. 1998). Inspection of both sides of all the slices of lung under an illuminated magnifying glass revealed small white foci (0.5–1 mm) that had not been observed in the fresh organ. The suspect lesions were sampled for histopathological examination, and Tb was recognised histologically in most of the badgers. This method provides the ideal selection of tissues for histopathological examination, but is time-

consuming and results in thin slices, which are difficult to process histologically. It is therefore mostly applicable to research settings.

Histopathology: Characteristics of NVL tuberculous lesions

The microscopic characteristics of tuberculous lesions vary according to several factors, such as the host species, the stage of development of the lesion, and the immunity of the host. The classical tuberculous granuloma, as described for cattle, is often not observed in carnivores. For instance, giant cells are very rare or do not occur in badgers (Gallagher and Clifton-Hadley 2000) and ferrets (Lugton *et al.* 1997). They were randomly distributed when present in lesions in possums (Cooke *et al.* 1995). In farmed mink, neither the typical organisation of the tuberculous granuloma nor Langhans giant cells were observed (Martino *et al.* 1986). Early lesions, such as aggregations of macrophages, do not have the structure of an organised granuloma and may be easily overlooked. In badgers, fibrotic calcified nodules with poor cellularity may be interpreted as a non-specific chronic lesion rather than a latent tuberculous focus. In deer, some tuberculous lesions may simply be accumulations of neutrophils surrounded by a fibrotic capsule, resembling an abscess caused by pyogenic bacteria (Rhyan and Saari 1995). In Ziehl-Neelsen-stained sections, AFO may be absent or few in number. Yet, even when the features of Tb characteristic for the particular species are identified histologically, and AFO are observed in the lesions, it is not possible to differentiate *M. bovis* from other mycobacteria.

Badgers

The microscopic characteristics of pulmonary lesions in badgers with NVL Tb were described by Gallagher *et al.* (1998). Two types of lesions were observed. In one, small active granulomas varied in their severity or stage of development. They were organised as a rounded epithelioid cell granuloma, with a small necrotic centre, containing debris and a few neutrophils, with macrophages and lymphocytes at the periphery. Giant cells were not observed. A mild fibroblastic proliferation encased the granulomas. A few AFO were observed in some of these lesions. Older granulomas had more extensive central necrosis, slightly more fibroplasia, and more AFO. Due to their small size, these lesions remain undetectable by eye or are easy to overlook. In the second, healed or regressed granulomas had clearly demarcated fibrotic capsules and contained a centre of coagulative necrosis and mild mineralisation (Figure 1). These lesions were ≤0.5 mm, and appeared to represent arrest or containment of infection. Some of the foci were slightly more cellular and had surrounding aggregates of lymphocytes, macrophages and a few degenerating epithelioid cells. Most of these lesions contained AFO; when present in the necrotic area, they were palely stained (Figure 2). The occurrence of early active granulomas containing many AFO were also reported adjacent to a healed, dormant focus. It was postulated that these satellite active granulomas resulted from extension of infection from the dormant focus.

Possums

Extensive histopathological studies demonstrated tuberculous lesions in a wide range of organs and tissues (Cooke *et al.* 1995). The smallest lesions, which were not detected macroscopically, consisted of aggregations of low numbers of large macrophages with angulated cytoplasmic boundaries, and even fewer lymphocytes (Figure 3). On examination of Ziehl-Neelsen-stained sections at low power, these cellular aggregates were paler than the surrounding tissue. AFO were not always found in small/early lesions.

Ferrets

Between 50–90% of tuberculous ferrets had microscopic granulomas in the liver, despite the lack of macroscopic lesions in that organ. They were randomly distributed in the hepatic parenchyma and were considered highly characteristic of Tb. The granulomas were formed by aggregations of macrophages, with fewer lymphocytes and plasma cells, and occasional neutrophils (Figure 4). In lymph nodes, aggregates of epithelioid cells, from a few to large sheaths of cells that replaced much of the lymph-node structure, were observed. Central necrosis surrounded by lymphocytes and plasma cells occurred in some of the epithelioid lesions. AFO were observed more commonly in epithelioid cells adjacent to necrotic areas. Mineralisation or giant cells were not recorded (Lugton *et al.* 1997).

Coyotes

A coyote with NVL Tb showed one focus of necrosis with mineralisation, containing AFO, in a mesenteric lymph node. In an infected coyote with enlargement of a mesenteric lymph node as the only macroscopic lesion, lymphoid hyperplasia was the only histological finding (Bruning-Fann *et al.* 2001).

Deer

In cases of NVL Tb in red deer, histology may reveal early granulomas or aggregations of macrophages in lymph nodes, mainly in the medial retropharyngeal (MP Martín-Hernando[1], pers. comm.) (Figure 5). In white-tailed deer, histological lesions in cases with NVL Tb were present only in oropharyngeal tonsils, and ranged from simple necrosis to caseation, suppuration, and formation of granulomas. AFO were found rarely (O'Brien *et al.* 2004). Likewise, early microscopic lesions were present in the tonsils of red deer with NVL Tb, and consisted of aggregations of macrophages (Figure 6).

European wild boar

An homogeneous increase in the size of lymph nodes was the only macroscopic change in *M. bovis*-infected wild boar (Martín-Hernando *et al.* 2007). Histologically, the lymph nodes and tonsils contained granulomas composed mostly of macrophages, sometimes also with other inflammatory cells and small (microscopic) areas of central necrosis (Figures 7 and 8). Most of these lesions were ≤0.5 mm in diameter.

The role of culture in the diagnosis of NVL Tb

Identification of *M. bovis* by culture from tissues taken post mortem is still the definitive 'gold standard' for estimating the true prevalence of Tb in wildlife hosts. However, the sensitivity of culture is affected by the protocol applied (Crawshaw *et al.* 2008). Mycobacterial culture of pools of lymph nodes was applied to a large-scale screening of Tb in mustelids, namely ferrets, stoats and weasels (*Mustela nivalis*), in New Zealand, and resulted in isolation of *M. bovis* from 1.25% of the ferrets. It was considered that culture would increase the capacity to demonstrate *M. bovis* infection in species such as ferrets in which lesions of Tb are often difficult to detect (de Lisle *et al.* 2008). Advantageously, the isolation of mycobacteria in cultures allowed further characterisation and classification of mycobacteria other than *M. bovis* and also the identification of the various genotypes of *M. bovis* isolated, using restriction endonuclease analysis (de Lisle *et al.* 2008). However, mycobacterial culture may be cost-prohibitive or difficult to perform in a field setting, or may be performed on pools of samples often from many individual animals, which may result in decreased sensitivity. For example, in a study of white-tailed deer

[1] MP Martín-Hernando, IREC, Ciudad Real, Spain

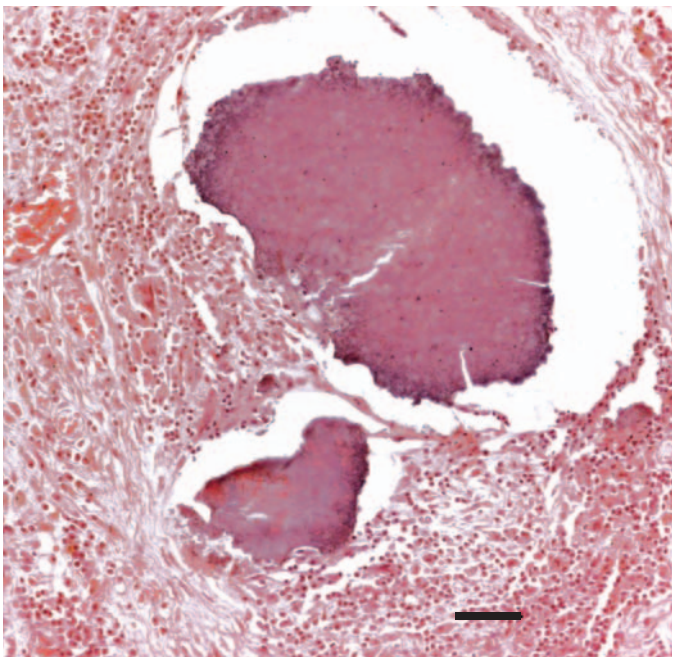

Figure 1. Light photomicrograph of a section of lung from a tuberculous badger with no macroscopic lesions, showing a healed or regressed granuloma, demarcated by a fibrotic capsule and with a centre of necrosis and mild mineralisation (H&E, bar=50 µm).

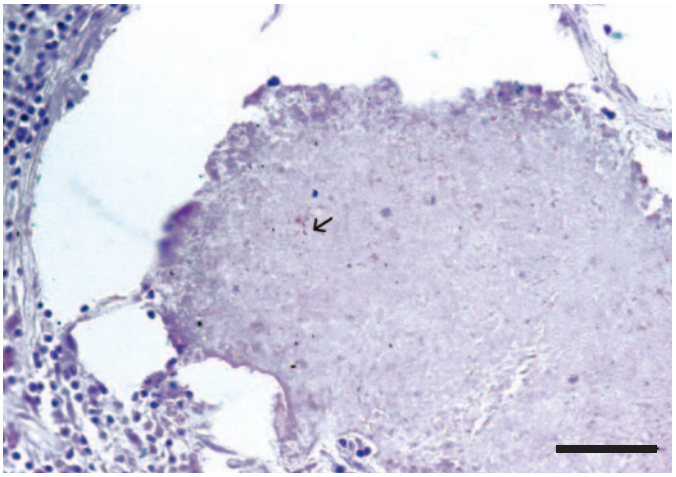

Figure 2. Light photomicrograph of a section of lung from a tuberculous badger. The same granuloma in Figure 1, showing the presence of acid-fast organisms (Ziehl-Neelsen, bar=50 µm).

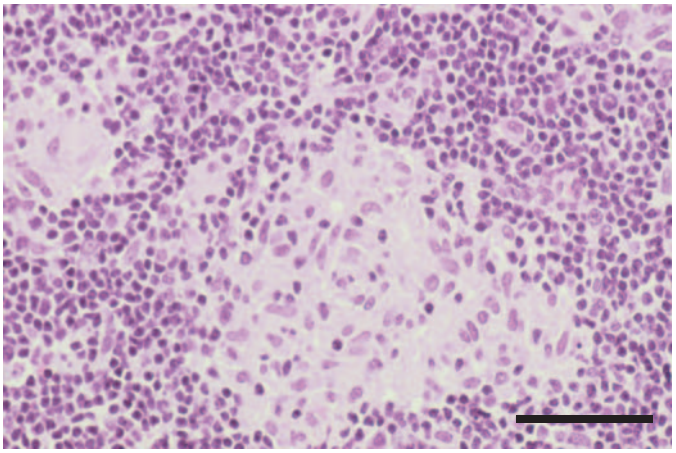

Figure 3. Light photomicrograph of a section of the deep axillary lymph node from a possum with no macroscopic tuberculous lesions, showing a small granulomatous focus comprising an aggregation of macrophages with angulated cytoplasmic boundaries (H&E, bar=50 µm).

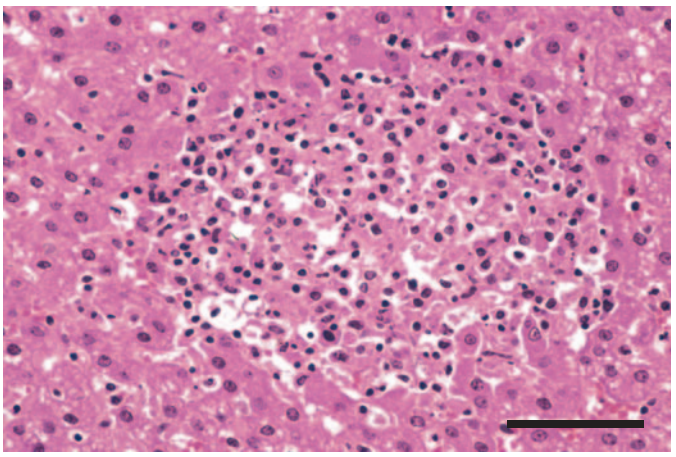

Figure 4. Light photomicrograph of a section of liver from a tuberculous ferret in which no macroscopic lesions were visible, demonstrating a small granulomatous focus (H&E, bar=50 µm).

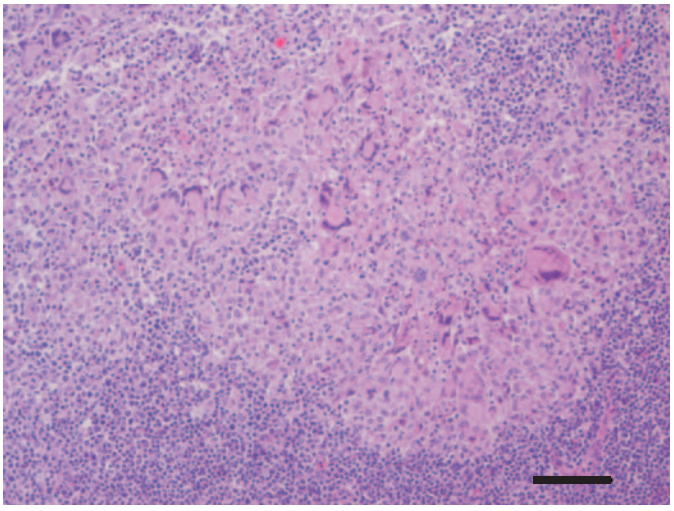

Figure 5. Light photomicrograph of a section of the medial retropharyngeal lymph node from a red deer with 'no visible lesion' tuberculosis, showing an aggregation of macrophages and numerous giant cells (H&E, bar=50 µm).

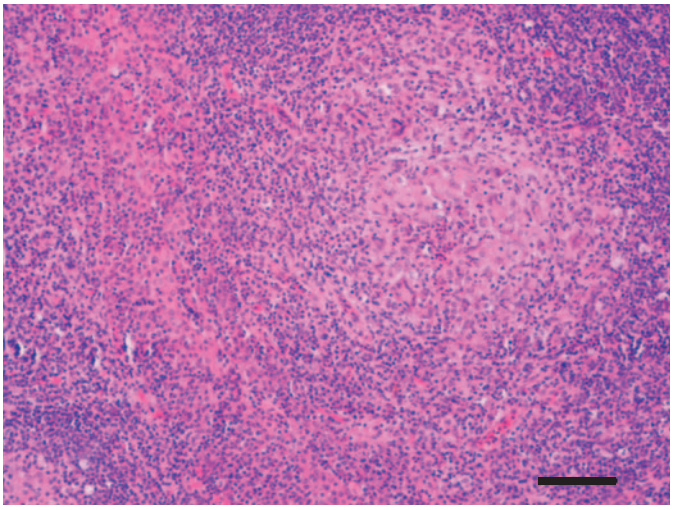

Figure 6. Light photomicrograph of a section of the oropharyngeal tonsil from a red deer with 'no visible lesion' tuberculosis, depicting an aggregation of macrophages (H&E, bar=50 µm).

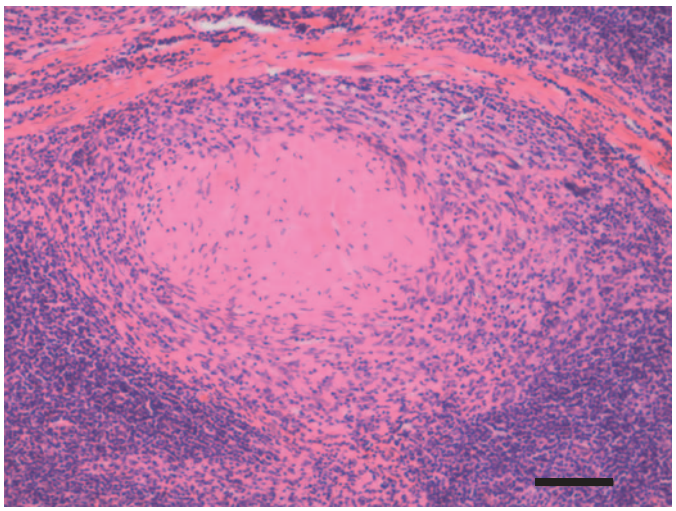

Figure 7. Light photomicrograph of a section of the mandibular lymph node from a European wild boar with 'no visible lesion' tuberculosis, showing an initial early-stage granuloma with central necrosis located close to a trabeculum, and a single giant cell in the periphery (H&E, bar=50 µm).

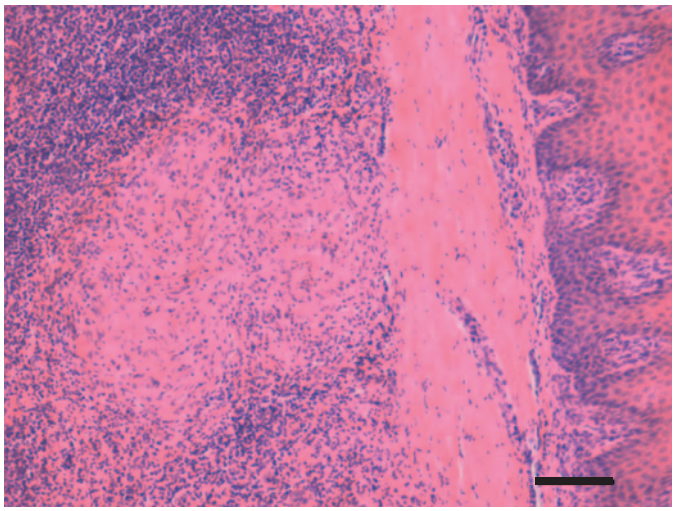

Figure 8. Light photomicrograph of a section of the oropharyngeal tonsil from a European wild boar with 'no visible lesion' tuberculosis, demonstrating an aggregation of macrophages close to the epithelium (H&E, bar=50 µm).

in Michigan, 1% of the deer without macroscopic lesions showed positive culture of *M. bovis* in a pool of lymph nodes from the head, and the oropharyngeal tonsils (O'Brien *et al*. 2004). The apparent prevalence of infection, i.e. the number of animals with macroscopic lesions consistent with Tb that were culture-positive, divided by the total number of animals examined, was considered to underestimate the true prevalence by 25%. Additionally, the study did not include the culture of other lymph nodes, such as mediastinal and mesenteric, and therefore likely further underestimated the true prevalence.

The role of PCR in the diagnosis of NVL Tb

The detection of organisms of the *M. tuberculosis* complex by the application of PCR methods to tissue is an attractive method as it is more rapid than conventional culture, and may be applied to formalin-fixed specimens (Miller *et al*. 1997). PCR also has the benefit of detecting bacterial DNA and therefore does not depend on the isolation of mycobacteria in a viable or culturable state. This has been demonstrated in humans in the case of *M. tuberculosis*, where bacterial DNA was detected in post-mortem lung tissue using *in-situ* PCR (Hernández-Pando *et al*. 2000). Those authors concluded that *M. tuberculosis* can persist intracellularly in lung tissue without histological evidence of tuberculous lesions. That the same may be true for *M. bovis* infection of animals is an intriguing possibility worthy of further investigation. Review of the literature revealed examples where PCR has been applied to the direct detection of *M. bovis* in tissue from animals. Sangster *et al*. (2007) applied the method to retropharyngeal, mesenteric and colonic lymph node tissue from 82 coyotes in conjunction with conventional culture, but failed to detect *M. bovis* by either method. The tissue had been previously frozen but was unfixed at the time of lysis for DNA extraction. In a survey of 781 free-ranging white-tailed deer by O'Brien *et al*. (2004), seven animals were confirmed as tuberculous by culture, in the absence of macroscopic lesions. Cranial lymph-node and tonsillar tissue from these animals was examined histologically, and subjected to PCR for the detection of *M. bovis*. Histopathological lesions consistent with Tb were found in all seven samples of tonsil but none of those from the lymph nodes. The investigators detected only one infected tissue sample (tonsil) by PCR. In that case, tissue had been frozen, then thawed, fixed in 10% buffered formalin and paraffin-embedded before an attempt was made to extract DNA for PCR. Whilst this method has yielded sensitivities of 81–93% in tissues from elk and cattle (Miller *et al*. 1997), O'Brien *et al*. (2004) considered that the poor sensitivity in their study might be due to repeated freezing and thawing of the tissue, the length of time it was held in storage, or the possibility that trimming of tissue samples for initial isolation may have removed a focal tuberculous lesion, or simply that PCR might be less sensitive on fixed compared with fresh tissue samples due to damage induced to chromosomal DNA by fixation then extraction (Heinmöller *et al*. 2001; Cataloluk *et al*. 2003). Therefore, whilst PCR methods have the potential to be more sensitive than culture or histopathology in detecting cases of Tb unapparent macroscopically, the methodology requires further investigation and optimisation before this potential might be realised.

NVL Tb and its epidemiological role: Shedding of *M. bovis*

Pathology studies can indicate the likelihood of mycobacterial shedding. For example, disseminated disease with extensive macroscopic lesions, with poor fibrotic containment of the granulomas, abundant AFO, and ulceration into the lumina of airways are conducive to aerogenous shedding (Gavier-Widén *et al*. 2001). Hosts of *M. bovis* that contaminate the environment or transmit infection directly to other animals of the same or other species generally show severe disease. However, this is not always the case. A recent study in badgers showed that only about 1% of adults had extensive severe lesions. The larger proportion of badgers with less severe lesions was considered responsible for the majority of the spread of infection (Jenkins *et al*. 2007). Epidemiologically, it is important to determine if NVL tuberculous animals shed mycobacteria, and via what route(s). It was found that 1/15 (7%) badgers with NVL Tb were detected as shedding *M. bovis* via the respiratory route (Gallagher *et al*. 1998).

Carnivores in general, with their apparent lower susceptibility to overt disease, their low rate of macroscopic lesions, and high rate of NVL Tb, are usually considered as spillover or dead-end hosts (O'Brien *et al*. 2006). However, any species of infected animals with NVL represent a pool of infected individuals, any one of which may develop more progressive or disseminated disease and become an excretor of *M. bovis*. The probability of that happening will be determined by many factors, but may include the host

species, anatomical site of infection, stress, starvation, or concomitant disease. Additionally, it is possible that scavenging carcasses of animals with NVL Tb could result in infection of individuals of the same or other species.

Possible factors related to the development of NVL Tb

The role of the strain of *M. bovis*

There is little information available on variability in the virulence characteristics of *M. bovis* isolates in different parts of the world. Such analysis is constrained by the level of differentiation possible with the method employed for molecular typing (Haddad *et al*. 2004), but on the basis of what has been observed for the closely-related *M. tuberculosis* there is good reason to suspect such variation does exist. For example, there is evidence that a higher proportion of isolates of *M. tuberculosis* from South India are of low virulence in guinea pigs (*Cavia porcellus*) compared with strains from Europe (Mitchison 1964).

Cattle challenged experimentally with *M. tuberculosis* strain H37Rv developed an NVL form of Tb, i.e. no lesions visible at post-mortem examination, despite the fact that the animals were infected successfully, based on tissue culture post mortem and conversion to a positive tuberculin test (H-M Vordermeier[2], pers. comm.). It is also evident that various mutant strains of *M. tuberculosis* can infect and proliferate in animal models without causing pathology. Recent studies with one such mutant revealed a major difference from the wild-type organism. In spite of having equal numbers of bacteria in infected sites, the mutant failed to produce granulomatous inflammation in either mice or guinea pigs (Hu *et al*. 2008). Hence, as single gene defects can give rise to this phenomenon in the laboratory there is no reason to consider that this does not happen in the wild, albeit at a lower frequency.

The host's natural resistance

Inherent resistance of the host to mycobacterial infection or disease progression has been partially recognised in mice (Vidal *et al*. 1993) and cattle (Qureshi *et al*. 1996). A variation of innate susceptibility to bovine Tb was described in farmed red deer. About 5–10% of the deer were highly resistant and the same percentage of individuals were highly susceptible. The genetic component of resistance was found to have a heritability of 0.48 (Mackintosh *et al*. 2000). High susceptibility was associated with poor early cellular immune response and high antibody levels within 6 weeks of infection, indicating an early change from Type 1 (protective) to Type 2 immune response. The genetic background associated with natural resistance to bovine Tb has been studied in European wild boar (Acevedo-Whitehouse *et al*. 2005; Naranjo *et al*. 2006ab), and to a lesser extent in red deer in Spain (Fernández-de-Mera *et al*. 2008). For example, two genes (complement component 3 and methylmalonyl-CoA mutase) were upregulated in non-tuberculous wild boar naturally exposed to *M. bovis*, and this may have contributed to limit infection or progression of the disease (Naranjo *et al*. 2007). In deer, the activity of genes expressing tight-junction proteins, interleukin-11R, bactenecin, CD62L, CD74, desmoglein, IgA and IgM differed between tuberculous and non-tuberculous but exposed individuals (Fernández-de-Mera *et al*. 2008). This suggests mechanisms by which host inflammatory and immune responses may be modulated, and may also explain differences in the proportion of individuals with NVL Tb.

The host species

Mycobacterium bovis infects a broad range of species; probably all mammals are susceptible to infection (Francis 1958). However, some hosts often develop severe forms of disease while others typically show mild or minimal lesions. For example, non-feline carnivores in general appear to develop a well-contained infection, with small or macroscopically unapparent lesions, and only a small proportion of the infected individuals show overt disease (de Lisle 1993; Delahay *et al*. 2002). It is also possible that the route of infection may determine the pathogenesis, and that the route of infection may be a feature of the species' behaviour and the epidemiology of the disease. For instance, most cases of Tb in dogs are due to infection with *M. tuberculosis* from close association with their owners (Une and Mori 2007). Also, some species may principally acquire infection through scavenging, e.g. ferrets (Caley and Hone 2002), whilst others apparently contract it mainly through aerosol transmission, e.g. badgers (Gallagher *et al*. 1976). Whilst differences related to species can be observed, the pathogenetic and immunological mechanisms that lead to these differences are not clear.

Latency, or dormancy, is a recognised feature of Tb in man, and has been recorded lasting for up to 60 years, when infection acquired in childhood finally recrudesced (Sjogren and Sutherland 1975). The NVL presentation in animals is likely to include such latent cases or merely early-stage infection. It is not known, however, if the infection in NVL Tb persists for life or may be transient, i.e. can be cured spontaneously, as is likely the case in a proportion of humans with *M. tuberculosis* infection (Ewer *et al*. 2006). It is likely that the vigour of the cellular reaction to the presence of the tubercle bacillus is an important determinant as to whether large visible florid lesions develop or minute lesions resulting in NVL Tb.

The host's age

The age of the animal may also influence the progression of infection. Young animals are known to develop more severe forms of tuberculous disease, a situation also encountered in humans (Rich 1951). Nolan (1991) found 35% of adult badgers had macroscopic tuberculous lesions, whilst in cubs the proportion was significantly higher at 65%. Under-detection of Tb was found to occur more frequently in adult badgers compared with cubs (Crawshaw *et al*. 2008). Similarly, yearling European wild boar had more severe tuberculous lesions than adults (Martín-Hernando *et al*. 2007).

Discussion

Some of the difficulties in diagnosing Tb in wild animals have been presented above. In order to estimate the true prevalence of Tb in wildlife hosts, a detailed and comprehensive post-mortem examination in combination with the detection and identification of *M. bovis* is needed. Obviously, the thoroughness of post-mortem techniques is very important in determining the rates of true NVL Tb and in providing information for Tb control, for example establishing whether the assumption that there is a large population of uninfected or non-infectious animals is in fact correct. However, a detailed post-mortem examination is often

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not feasible because large numbers of animals may need to be examined frequently over a short time period and under field conditions. Necropsy techniques are not always standardised or adapted to a particular species and/or disease presentation. The extent of the underestimate of the prevalence of Tb when based on macroscopic findings only depends, among other factors, on the prevalence of NVL Tb in the species. Furthermore, meat inspection fails to detect Tb in NVL cases in production species, which therefore enter the food chain (Lugton *et al*. 1998; Vicente *et al*. 2006). All these factors may result in the actual prevalence of Tb being underestimated, or biased towards well-established disease. This was the case in early prevalence studies in feral ferrets and brushtail possums in New Zealand, that were limited to field post-mortem examinations (Coleman 1988; Pfeiffer *et al*. 1995; Ragg *et al*. 1995b). Only well-developed lesions or end-stage disease were recognised, and a significant number of less-advanced cases went undetected (Lugton *et al*. 1997).

These points were exemplified recently in a study of badgers, where the estimate of the prevalence of Tb was nearly doubled through the use of a detailed post-mortem protocol in which more tissues were examined, together with a combination of more extensive culture supplemented with histopathology (Crawshaw *et al*. 2008). Whilst those authors conceded that such extensive post-mortem examination was unlikely to be practical on a routine basis, it nonetheless was valuable in helping gauge the true prevalence of infection, and provided valuable information to help refine future post-mortem protocols, e.g. by indicating which tissues should be targeted for examination. A protocol developed for thorough post-mortem examinations of possums (Coleman *et al*. 1994) has been successfully applied, sometimes with slight modifications, in many field studies by other researchers to investigations of Tb in possums (Jackson *et al*. 1995) and other species (Lugton *et al*. 1997). Similar approaches could be advocated for other species where equivalent information is lacking.

In spite of this, studies of Tb in wildlife populations based on post-mortem examination alone may still be beneficial, particularly in situations in which large numbers of animals are examined. For example, post-mortem examination was considered to provide valuable information applied to exploring the magnitude and geographical distribution of infection in a large-scale study of red deer and wild boar from Spain, given the large number of samples that were obtained from an extensive area (Vicente *et al*. 2006, 2007).

Different diagnostic methods have different sensitivities and specificities. There will also be differences in the results obtained when the same basic methods are undertaken to different degrees. Hence, in the first instance, when several diagnostic methods are applied to the same animal, there may be disagreements in the results obtained by each method. This situation is more likely to be encountered in mycobacterial infections where there are no or few macroscopic lesions, indicating early infection or that the infection has not progressed (Nielsen and Toft 2008). In early infection, there may be few bacilli in tissues and they may not be distributed evenly throughout the tissue so that differences may arise by chance. In the case of latency, mycobacteria may be present but refractory to culture (Shleeva *et al*. 2002). Possible scenarios include the detection of visible lesions consistent with Tb, and AFO observed histologically, but no mycobacteria isolated on culture; or no lesions seen in culture-positive cases. For example, in a detailed study of 132 badgers, evidence of Tb was seen in 66 (50%). Of those diagnosed as having Tb, 57 (86%) were identified as tuberculous by culture, 49 (74%) had histological lesions consistent with Tb, including nine that were culture-negative, and 28 (42%) had only macroscopic lesions (LAL Corner[3], pers. comm.).

In conclusion, *M. bovis* infection may not be accompanied by the presence of macroscopic lesions, a feature often observed in some wildlife reservoirs or accidental hosts, making the diagnosis in these species difficult. Highest sensitivity is achieved when several diagnostic methods are applied to the same animal, including thorough standardised post-mortem examination, histopathology and mycobacterial culture, including culture of lymph nodes (sometimes pooled) and/or oropharyngeal tonsils from animals with no macroscopic lesions. In situations in which the practical and economical limitations preclude such diagnostic applications to all/individual animals, knowledge of the prevalence of NVL Tb in the population is necessary for more accurate estimates. At a very minimum, the diagnostic methods applied for studying the prevalence of Tb in the population should be clearly described, to at least allow comparison among studies.

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