

## BRUCELLOSIS IN MARINE MAMMALS, WITH SPECIAL EMPHASIS ON THE REPUBLIC OF CROATIA

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### Summary

After almost two decades of research and evidence of brucellosis in marine mammals, it has become widely known that this disease is prevalent in marine mammals on a global scale. Positive animals have been found in the northern Atlantic, Mediterranean Sea and in the Arctic, including the Barents Sea. Infected or exposed animals have been detected along the Atlantic and Pacific coasts of North America, off the coasts of Peru, Australia, New Zealand, Hawaii, the Solomon Islands, and in the Antarctic Ocean. Brucellosis most commonly occurs in the harbour porpoise (*Phocoena phocoena*), followed by the striped dolphin (*Stenella coeruleoalba*), Atlantic white-sided dolphin (*Lagenorhynchus acutus*), bottlenose dolphin (*Tursiops truncatus*), short-beaked common dolphin (*Delphinus delphis*) and minke whale (*Balaenoptera acutorostrata*). A positive serological reaction has been confirmed in 35 species of whales and 14 species of seals, two subspecies of sea otters, one species of freshwater otter and the polar bear. To date, brucellosis has been found in marine mammals in the Mediterranean Sea in Italy and Spain. The first research on brucellosis in dolphins in the Republic of Croatia began in 2015. *Brucella* sp. was isolated from the lymph node of a bottlenose dolphin found dead in the Poreč region. This was the first evidence of brucellosis in dolphins in the Adriatic Sea. The isolated sample was identified as *Brucella ceti* strain ST27, making this the first record of this strain in Europe, which may present a significant threat to human health.

**Keywords:** marine mammals; dolphin; brucellosis; *Brucella ceti* ST27; Croatia.

### INTRODUCTION

Brucellosis has been identified and proven in whales, dolphins, seals, sea lions, walrus and otters. This disease appears to be widely distributed in marine mammals, and the clinical signs include placentitis and miscarriage, neonatal mortality, meningoencephalitis, and skin abscesses. Potential hosts for *Brucella* sp. in aquat-

ic systems may consist of some 130 species of marine and freshwater mammals that live and feed in the seas, rivers and lakes. This includes 86 different species of whales of the order Cetacea, suborder Mysticetia (baleen whales) and suborder Odontocetia (toothed whales) that also comprises the dolphins, 36 species belonging to the ordo Carnivora, suborder Pinnipedia (seals), including the families *Otariidae* (eared seals), *Odobenidae* (walruses), and *Phocidae* (seals). Other mammals that may be infected include the sea otter (*Enhydra lutris*), marine otter (*Lutra felina*), polar bear (*Ursus maritimus*), manatee (*Trichechus* sp.), and dugong (*Dugong dugong*). Natural infection has also been recorded in humans, primarily those professionally exposed to marine mammals [1].

## ETIOLOGY

*Brucella* (*B.*) sp. was first isolated in 1994 from the carcasses of harbour seal (*Phoca vitulia*), harbour porpoise (*Phocoena phocoena*) and short-beaked common dolphin (*Delphinus delphis*) beached along the coast of Scotland, and from the aborted young of a bottlenose dolphin in captivity in California [2,3]. Initially, all strains were called *B. maris* [4]. Later studies indicated there were in fact two species of *Brucella cetacean*. Isolates from whales were initially called *Brucella cetacean* [5]. In 2007, they were renamed *Brucella ceti*, as the natural hosts were confirmed to be the whales (cetaceans). Isolates from seals differed from *B. ceti* and were called *B. pinnipedialis*, with seals (*Phocidae*) as the natural hosts [6-9].

## TRANSMISSION OF BRUCELLOSIS IN MARINE MAMMALS

The route of transmission of brucellosis among marine mammals has not yet been fully elucidated. It is believed that infections in whales may be transmitted sexually, or in contact with aborted off spring and placental tissue. Vertical transfer from mother to offspring is known [10-12]. In seals, the route of transmission is insufficiently known, though their communal lifestyle and constant direct contact between animals spurs the spread of infection. Lung nematodes (*Pseudalius inflexus*) are considered as possible vector for brucellosis in the marine environment. *Brucella* has been found in the reproductive organs and intestinal lumen of lung nematodes isolated from dolphins and seals. In their lifecycle, parasites migrate through the respiratory system of sea lions, enter into the digestive system and move into the environment through excrement. The opaleye fish (*Girella nigricans*) and other intermediaries, such as coprophagous fish, eat the contaminated faeces. These fish are later consumed by marine mammals, and the larvae are released in the digestive tract of the mammals, when they migrate into the lungs and the cycle continues [13].

## DISTRIBUTION OF BRUCELLOSIS IN MARINE MAMMALS

After almost two decades of research and evidence of brucellosis in marine mammals, it has become well known that this disease is globally distributed in marine mammals. Bacteriologically or serologically positive animals have been found in the Northern Atlantic, Mediterranean Sea and in the Arctic, including the Barents Sea. Infected or exposed animals have been found along the Atlantic and Pacific coasts of North America, the coast of Peru, Australia, New Zealand and Hawaii, on the Solomon Islands, and in the Antarctic [14-17]. The most recent research has also proven the presence of brucellosis in the bottlenose dolphin in Croatia, in the northern Adriatic Sea [18].

Specific comparative analyses have shown that certain species of whales and dolphin are more susceptible to *Brucella* infection than others. Among the toothed whales, brucellosis most often affects the harbour porpoise (*Phocoena phocoena*), striped dolphin (*Stenella coeruleoalba*), Atlantic white-sided dolphin (*Lagenorhynchus acutus*), bottlenose dolphin (*Tursiops truncatus*) and short-beaked common dolphin (*Delphinus delphis*) and minke whale (*Balaenoptera acutostrata*). The average incidence of brucellosis in marine mammals varies among species. Research has been conducted in different geographic areas on different sample sizes. To date, about 4% of the investigated marine mammals have been found to be serologically positive in the Atlantic, Pacific and Arctic Oceans. In less comprehensive studies, seroprevalence ranges from 0 to 80%. Positive serological reactions have been confirmed in 35 whale species and 14 seal species, two sea otter subspecies, one freshwater otter species, and in the polar bear [1,19].

### BRUCELLA CETI

*B. ceti* has been isolated from both suborders of whales: Mysticeti, the baleen whales, and Odontoceti, the toothed whales. The first suborder includes four families of baleen whales, which feed by filtering plankton (rorquals) and skim feeding (right and pygmy whales), or by filtering the silt off the seabed (grey whales). The suborder of toothed whales includes a group of nine families, including the sperm whales and pygmy sperm whales, beaked whales, dolphins and porpoises, as well as the Indian and South American river dolphins. *B. ceti* has been determined (by isolation or PCR) in only four families: *Balaenopteridae* (rorquals), *Phocoenidae* (porpoises), *Delphinidae* (dolphins, killer whales, pilot whales and relatives) and *Monodontidae* (belugas and narwhals). *Brucella* has been proven in many species, such as the harbour porpoise (*Phocoena phocoena*), bottlenose dolphin (*Tursiops truncatus*), striped dolphin (*Stenella coeruleoalba*), white-beaked dolphin (*Lagenorhynchus albiros-*

tris), killer whale (*Orcinus orca*) and others. Antibodies have been proven in seven families and 35 different species, suggesting the conclusion that *Brucella* infections in marine mammals in the whale families is very common [3,15,20,21].

The majority of isolates have been derived from whales beached on the shores of the Atlantic Ocean in North America and Europe, and on the southwestern and eastern coasts of the Pacific Ocean. Isolates in Europe are primarily from the Northern Atlantic and North Sea off Scotland, Spain, England and Wales. In the western Atlantic, *Brucella* strains have been isolated in mammals off the coasts of New England and the entire eastern seaboard of the United States, and in the Gulf of Mexico. *Brucella* strains have also been isolated in the eastern and southwestern Pacific [22-24]. *Brucella* infections in marine mammals are globally distributed [25]. Infections with the species *B. ceti* are specific to the nervous system, and have been described in two cetacean species: striped dolphin (*Stenella coeruleoalba*) in Europe and off the coast of Costa Rica, and in the Atlantic white-sided dolphin off the coast of Great Britain. Though all the strains isolated from whales belong to the species *B. ceti*, three different groups of strains are known based on their preferred host, certain bacteriological properties, and specific genetic traits: the strains isolated from short-beaked common dolphin (ST23), from the harbour porpoise (ST26) and from humans (ST27). It is believed that the strains isolated from the dolphins and porpoises are variable in experiments on terrestrial animals, and are not infectious for humans. However, brucellosis has proven to be a highly chronic disease in certain common dolphins and harbour porpoises, with severe clinical signs and pathological signs relating to abortions, male infertility, neurobrucellosis, cardiopathology, bone lesions and skin, beachings and ultimately in death [22].

The presence of antibodies has been proven in 58 serum samples originating from toothed whales caught in Peru in the period from 1993 to 1995 and from 24 whales beached along the Spanish coast in the period from 1997 to 1999. Positive serological reactions were confirmed in 21 (77.8%) of 27 samples originating from the dusky dolphin (*Lagenorhynchus obscurus*), in 3 (50%) of 6 samples of long-beaked common dolphin (*Delphinus capensis*), in 8 (35%) of 23 samples of bottlenose dolphin (*Tursiops truncatus*), in 5 (25%) of 20 Burmeister's porpoise (*Phocoena spinipinnis*) and in 2 (33%) of 6 samples of striped dolphin (*Stenella coeruleoalba*) [24]. Off the Pacific coast of Costa Rica, *B. ceti* was isolated from 6 striped dolphins (*Stenella coeruleoalba*) [11]. In the period from 2008 to 2011, a total of 112 harbour porpoises (*Phocoena phocoena*) were found dead off the Dutch coast, and these animals were tested for brucellosis. Of these animals, 5 (4.5%) were positive for *Brucella* sp. Molecular analysis showed that the strain was *B. ceti* ST 23 [43]. *B. ceti* has also been isolated from harbour porpoises (*Phocoena phocoena*) washed up on the beaches of Belgium [26].

## BRUCELLA PINNIPEDIALIS

*B. pinnipedialis* has been isolated from seals of the families *Phocidae* (seals) and *Otariidae* (sea lions). Six species of seals have been found to harbour this disease: hooded seal (*Cystophora cristata*), ringed seal (*Phoca hispida*), harp seal (*Phoca groenlandica*), grey seal (*Halichoerus grypus*), harbour seal (*Phoca vitulina*). This species of *Brucella* has also been isolated from the California sea lion (*Zalophus californianus*). Antibodies have been found in 17 different marine mammal species from the order Carnivora, subordo Pinnipedia (families *Otariidae*, *Odobenidae*, *Phocidae*) and subordo *Fissipedia* (families *Mustelidae* and *Ursidae*), steller sea lion, Australian sea lion, Antarctic fur seal, South American fur seal, walrus, harbour seal, Pacific harbour seal, harp seal, grey seal, Hawaiian monk sea lion, hooded seal, leopard seal, Weddell's seal, Alaskan sea otter, California sea otter, European otter, and the polar bear. Isolates have been found from animals in the northern Atlantic (Northern Ireland, New England, Canada), the North Sea (Scotland, Germany), and the Pacific Ocean (California, USA). *B. pinnipedialis* has been isolated from hooded seals or from beached animals or seemingly healthy individuals captured in their natural environment [1,27,28]. Following the isolation of *Brucella* from porpoises and whales in Scotland and in Northern England, serological testing was also conducted on other marine mammals that could be exposed to *Brucella* infections along the coasts of England and Wales. In the period from 1989 to 1995, 153 serum samples were collected from marine mammals. Positive serological reactions were recorded in 6 (10%) of 62 grey seals (*Halichoerus grypus*), in 1 (8%) of 12 harbour seals (*Phoca vitulina*), in 11 (31%) of 35 samples of harbour porpoises (*Phocoena phocoena*) and in 9 (20%) of 44 samples of short-beaked common dolphins (*Delphinus delphis*) [29]. Antibodies for *B. pinnipedialis* were most often found in common seals (*Phoca vitulina*) in 3 (14 %) of 21 samples and in 4 of 53 (8%) harp seals (*Phoca groenlandica*). A particularly high incidence of serologically positive animals (35%) was confirmed in the hooded seal (*Cystophora cristata*) in the northern part of the Atlantic Ocean and Barents Sea. In 49% of tested samples of common seal from Scotland, brucellosis antibodies were confirmed, as in 21% of samples from North America and 75% of tested samples of sea lions from Australia [23]. Brucellosis antibodies were detected in 35% of samples of hooded seals (*Cystophora cristata*), in 2% of harp seals (*Phoca groenlandica*) and in 10% of ringed seals (*Phoca hispida*). Research has shown that brucellosis is widely distributed among marine mammals in the northern Atlantic [30]. Antibodies were confirmed in 16 (5.4%) of 297 tested serum samples in polar bear (*Ursus maritimus*) from the Svalbard islands and in the Barents Sea. Antibodies were found in the same area in ringed seal (*Phoca hispida*) and harp seal (*Phoca groenlandica*), and these

seal species represent an important dietary source for bears, and as such could be an important source of infection [31]. Brucellosis was confirmed in 11 (38%) of 29 seemingly healthy hooded seals (*Cystophora cristata*) captured in their natural habitat in the northern Atlantic Sea and Greenland [28]. Brucellosis antibodies were confirmed in polar bear (*Ursus maritimus*) in Alaska, in the range of 6.8% to 18.5% of processed samples [32].

## DISTRIBUTION OF BRUCELLOSIS IN MARINE MAMMALS IN THE MEDITERRANEAN SEA

In the Mediterranean Sea, brucellosis has been confirmed in marine mammals in Italy, Spain and Croatia (Figure 1). *Brucella sp.* was first isolated in the Mediterranean Sea from the brain, lungs and lymph nodes of the striped dolphin (*Stenella coeruleoalba*) in Italy in 2012 in the Tyrrhenian Sea (off the coast of Tuscany) (Figure 1, number 1). This isolate was classified as *Brucella ceti* type 1, and multi-locus sequencing (MLST) categorised it as belonging to the strain *B. ceti* ST 26 [33]. Other described cases of brucellosis were in two striped dolphins (*Stenella coeruleoalba*) washed up in southern Italy, on the Ionian coast of the Mediterranean Sea. Brucellosis was isolated from dolphins in the area of Galipolli Lido Pizzo in March 2012 and again in November 2012 from dolphins in the area Porto Cesareo Bacino. Both isolates were molecularly confirmed to belong to the strain *B. ceti* ST 26 [19]. (Figure 1, number 2). Brucellosis was confirmed in two striped dolphins (*Stenella coeruleoalba*) found on the shores of Catalonia in Spain (Salou and Badalona areas) and in the bottlenose dolphin (*Tursiops truncatus*) (Qunit) in Spain. Two striped dolphins were found alive, one with signs of neurobrucellosis, including reduced mobility, side swimming and a lack of coordination, while the second showed no clinical symptoms. The corpse of a bottlenose dolphin was soon found washed ashore. The organs tested from all three dolphins contained *B. ceti*, and MLST testing categorised the samples as belonging to the strain *B. ceti* ST 26 [34], (Figure 1, number 3).

## BRUCELLOSIS IN MARINE MAMMALS IN THE REPUBLIC OF CROATIA

The bottlenose dolphin (*Tursiops truncatus*) is widely distributed in the Mediterranean Sea. It is the only permanent resident in the Croatian waters of the Adriatic Sea. This species is associated (resident) to the area it lives in, creating communities of varying size. Other cetaceans found occasionally in the Adriatic Sea are the striped dolphin (*Stenella coeruleoalba*), Risso's dolphin (*Grampus griseus*), and Cuvier's beaked whale (*Ziphius cavirostris*). Considering the small area and closed nature of



**Figure 1.** Distribution of brucellosis in dolphins in the Mediterranean Sea. In Italy (numbers 1 and 2), Spain (3) and Croatia (4).



**Figure 2.** A dead bottlenose dolphin (*Tursiops truncatus*) beached on the shores of the Adriatic Sea.

the Adriatic Sea, coupled with strong human population pressures, particularly from the summer tourism season, dolphins in the Adriatic face numerous threats. In Croatia, 50% of all dolphin deaths are from anthropogenic causes, including capture or entanglement in fishing nets, intentional deaths by fishermen, or killings for other reasons. Some animals also die of natural causes (*Figures 2,3 and 4*).

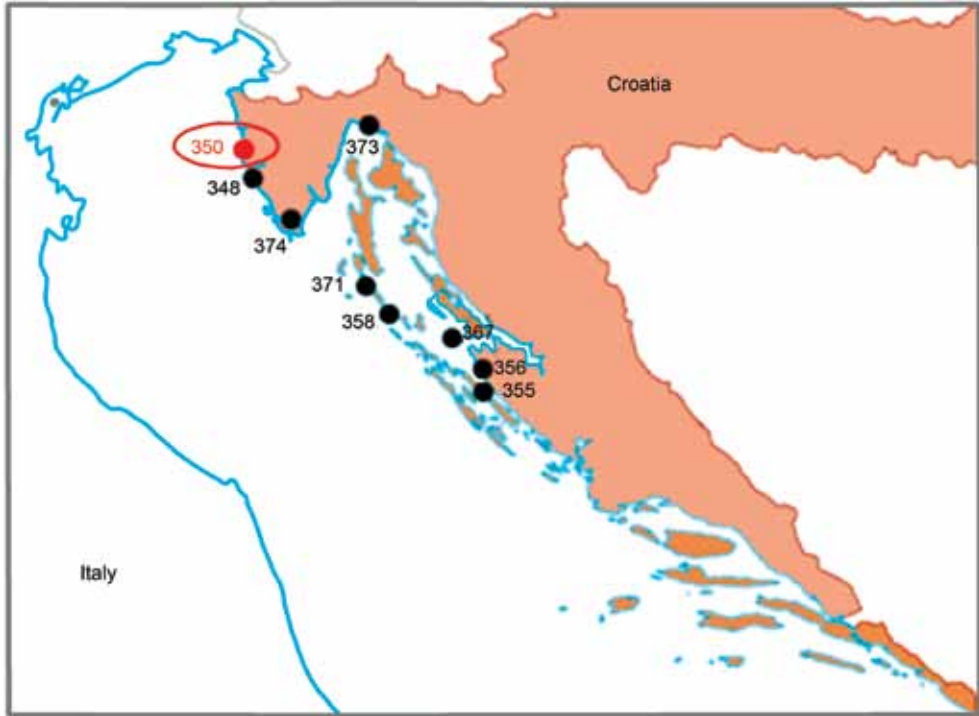


**Figure 3.** A dead striped dolphin (*Stenella coeruleoalba*) beached on the shore of the Adriatic Sea.



**Figure 4.** Corpse of a dead bottlenose dolphin. It is evident that the dead dolphins have no fluke, likely they were cut off after entanglement in a fishing net.





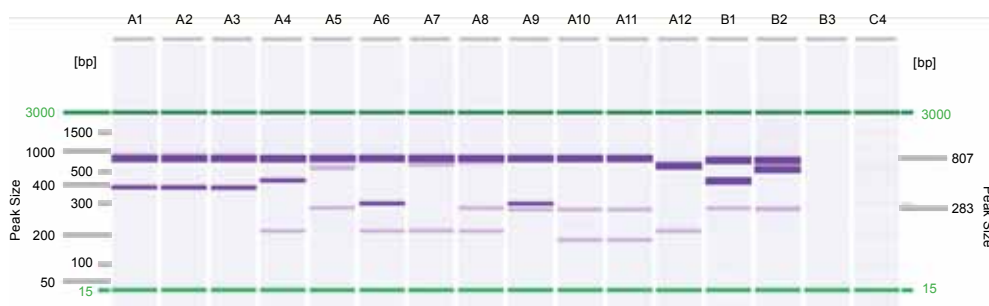
**Figure 5.** An overview of sites in the Croatian part of the Adriatic Sea where bottlenose dolphin corpses were found and tested for brucellosis.



**Figure 6.** Bottlenose dolphin (*Tursiops truncatus*) no. 350 prior to posmortal examination the Department of Anatomy, Histology and Embriology, Faculty of Veterinary Medicine, University of Zagreb.

The first research on brucellosis in dolphins in the Republic of Croatia began in summer 2015. During 2015 and 2016, a total of 9 dolphins found dead at the following locations were tested: Rovinj (348), Poreč (350), Ošljak (355), Molat (356), Lošinj (358), Vir (367), Lošinj (371), Rijeka (373), Pula (374), (Figure 5). *Brucella* sp. was isolated from the lymph nodes of bottlenose dolphin no. 350, found near Poreč (Figure 6).

Isolate (no. 350) was isolated on the fifth day after inoculation on Farrell agar at 37°C. The available molecular methods confirmed it belonged to the genus *Brucella*. Conventional PCR methods confirmed the presence of the gene BCSP-31, and 100% homology with known *Brucella* species, including species isolated from marine mammals based on the 16S rDNA sequences of [35,36]. Identification of the species



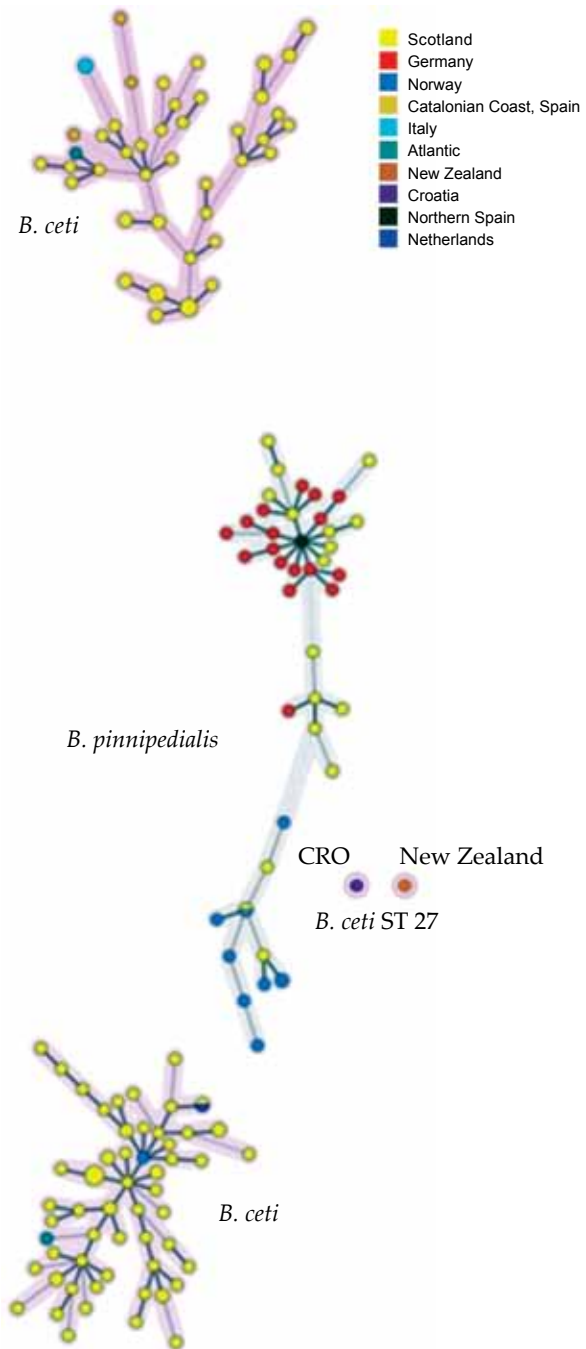
**Figure 7.** Multiplex - suis (Suis - ladder) (A1 - strain 350/1, A2 - strain 350/2, A3 - strain 350/3, A4 - *B.suis* referential strain (r.s.) bv.1, A5 - *B.suis* r.s. bv.2, A6 - *B.suis* r.s. bv.3, A7 - *B.suis* r.s. bv.4, A8 - *B.suis* r.s. bv.5, A9 - r. s. *B. abortus* S99, A10 - r.s. *B. melitensis* 16M, A11 - r.s. *B. ovis* REO 198, A12 - r.s. *B. canis* RM 666, B1 - *B. pinnipedialis* soj, B2 - *B.ceti* soj, B3 - negative control, B4 - size marker).

within the genus was conducted using multiplex PCR known as the Bruce-ladder method [37]. Other multiplex PCR methods such as Suis-ladder also used in order to identify the correct *Brucella* species [38], (Figure 7).

With the aim of differentiating the species *B.ceti* and *B. pinnipedialis*, additional primers were used [34,38,39]. Correct species could still not be determined with certainty. Therefore, further genotyping analyses of the strain were necessary for the identification of the number of tandem repeats on 16 gene loci, MLVA method [40,41], and sequence analysis of nine gene loci MLST method [42]. These methods gave more detailed phylogenetic information and important epidemiological data. Only MLST method gave the final determination that this strain was *Brucella ceti* ST27. This is the first record of brucellosis in a dolphin in the Adriatic Sea, and the first record of the strain ST27 in Europe, which also presents a human health risk [18], (Figure 8).

**DIAGNOSTICS,  
TREATMENT AND  
SUPERVISION OF  
BRUCELOSIS IN MARINE  
MAMMALS**

There are no specific clinical symptoms that would indicate brucellosis in marine mammals. The disease may be suspected in cases of miscarriage or pathological findings of orchitis, epididymitis, subcutaneous skin abscesses, meningitis or meningoencephalitis, or in the case of certain systematic diseases. Brucellosis has also been isolated from the organs of healthy animals. In order to better understand the pathogenesis, epizootiology and epidemiology of brucellosis, sources of infections in marine



**Figure 8.** Minimum Spanning Tree (MST) depiction of results of the MLST method on the isolated strain 350 in comparison with strains available in the Brucella2012 database. Strains are marked as belonging to the species *B. ceti* or *B. pinnipedialis* and origin, in which the colours listed in the legend represent countries.

mammals in the oceans and seas around the world, it is necessary to perform objective diagnostics and, wherever possible, to isolate and identify *Brucella* strains. Attempts to treat dolphins in captivity using antibiotics have proven to be ineffective. Due to the long-standing intracellular survival of *Brucella* in the body and the risk of the spread of infection to other animals and humans, it is considered essential and justified to properly dispose of positive animals, particularly those with clinical complications. There is no systematic supervision system for brucellosis in marine mammals, either for living animals or for those found dead or beached. Also human infections and spread of the disease are possible. The meat and organs for further consumption are rarely tested. Most often, marine mammals are captured and processed without any additional precautionary measures. Both species, *B. ceti* and *B. pinnipedialis*, have all the properties of infectivity, and can infect humans or other animal species [43].

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## Sažetak

**Bruceloza u morskih sisavaca s posebnim osvrtom na Republiku Hrvatsku**

Nakon gotovo dva desetljeća istraživanja i dokaza bruceloze u morskih sisavaca, opće je poznato da je bruceloza u morskih sisavaca globalno rasprostranjena. Pozitivne životinje pronađene su u sjevernom Atlantiku, Sredozemnom moru i na Arktiku, uključujući i Barentsovo more. Zaražene ili zarazi izložene životinje otkrivene su duž atlanske i pacifičke obale Sjeverne Amerike, obale Perua, Australije, Novog Zelanda, Havaja i na Solomonskim otocima te Antarktici. Bruceloza se najčešće javlja u obalnih dupina (*Phocoena phocoena*), zatim u plavobijelog dupina (*Stenella coeruleoalba*), atlanskog bjelobokog dupina (*Lagenorhynchus acutus*), dobrog dupina (*Tursiops truncatus*) i običnog dupina (*Delphinus delphis*) te patuljastog kita (*Balaenoptera acutostrata*). Pozitivne serološke reakcije utvrđene su u 35 vrsta kitova i u 14 vrsta perajara, dvije podvrste morskih vidri, jedne vrste slatkovodnih vidri i polarnog medvjeda. Do sada je na području Sredozemnog mora bruceloza u morskih sisavaca dokazana u Italiji, Španjolskoj. Prva istraživanja bruceloze u dupina u Republici Hrvatskoj započela su u ljeto 2015. godine. *Brucella* sp. izdvojena je iz limfnih čvorova dobrog dupina koji je uginut nađen na području Poreča. To je prvi dokaz bruceloze dupina u Jadranskom moru. Izdvojen je i identificirana *Brucella ceti* soj ST27 koji je prvi puta dokazan u Europi, što može predstavljati značajnu prijetnju zdravlju ljudi.

**ključne riječi:** morski sisavac; dupin; bruceloza; *Brucella ceti* ST 27; Hrvatska.

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