Article

Not peer-reviewed version

Brucella ceti in Common Dolphins (*Delphinus delphis*) in Portugal – Characterization of First Isolates

Sandra Cavaco , Miguel L. Grilo , Ricardo Dias , Mónica Nunes , Pedro Pascoal , Marcelo Pereira , Catarina Fogaça , Ana Beatriz Costa , Sofia Pardal , Ana Cristina Ferreira *

Posted Date: 9 December 2024

doi: 10.20944/preprints202412.0676.v1

Keywords: Atlantic Sea; Brucella ceti; Cetaceans; Comparative Genomics; Infection surveillance; Marine environment health; Portugal

 doi:10.20944/preprints202412.0676.v1

Article

Brucella ceti in Common Dolphins (*Delphinus delphis*) in Portugal–Characterization of First Isolates

Sandra Cavaco [1], Miguel L. Grilo [2,3,4], Ricardo Dias [5,6], Mónica Nunes [5], Pedro Pascoal [5], Marcelo Pereira [6], Catarina Fogaça [2], Ana Beatriz Costa [2], Sofia Pardal [2] and Ana Cristina Ferreira [1,6,7,*]

1 National Institute for Agrarian and Veterinary Research (INIAV IP), Av. da República, Quinta do Marquês, 2780-157 Oeiras, Portugal
2 RALVT – Rede de Arrojamentos de Lisboa e Vale do Tejo, ISPA–Instituto Universitário de Ciências Psicológicas, Sociais e da Vida, Rua Jardim do Tabaco 34, 1149-041 Lisboa, Portugal
3 Egas Moniz Center for Interdisciplinary Research (CiiEM), Egas Moniz School of Health & Science, Campus Universitário, Quinta da Granja, 2829-511 Almada, Portugal
4 MARE–Marine and Environmental Sciences Centre/ARNET–Aquatic Research Network, ISPA–Instituto Universitário de Ciências Psicológicas, Sociais e da Vida, Rua Jardim do Tabaco 34, 1149-041 Lisbon, Portugal
5 cE3c—Centre for Ecology, Evolution and Environmental Changes & CHANGE—Global Change and Sustainability Institute, Faculdade de Ciências, Universidade de Lisboa, Campo Grande, 1749-016 Lisboa, Portugal
6 BioISI-Biosystems and Integrative Sciences Institute, Faculty of Sciences, University of Lisbon, 1749-016 Lisboa, Portugal
7 I-MVET-Faculty of Veterinary Medicine, Lusófona University, University Centre of Lisbon, 1749-024 Lisbon, Portugal
* Correspondence: cristina.ferreira@iniav.pt

Simple Summary: This study investigates *Brucella ceti* infections in marine mammals stranded along the Lisbon and Tagus Valley coast from 2022 to mid-2024, reporting the first evidence of this zoonotic agent in Portuguese waters. Among 59 animals, *B. ceti* was found in 5.1% of common dolphins using cultural methods, though PCR testing revealed a higher infection rate (23.7%), highlighting challenges in accurate prevalence estimation. One dolphin displayed neurobrucellosis-like symptoms, while others had varied clinical signs, complicating diagnosis. Genetic analysis linked these isolates to other Atlantic *B. ceti* strains, suggesting dolphin-specific lineages. The findings emphasize the need for ongoing research and better diagnostic strategies to address the risks of *B. ceti* in marine ecosystems.

Abstract: This study investigates *Brucella ceti* infection in marine mammals stranded along the Lisbon and Tagus Valley coast between 2022 and mid-2024, marking the first report of *Brucella* presence in Portuguese waters. Out of 59 examined marine mammals, *B. ceti* was isolated in three common dolphins (5.1%), a prevalence rate consistent with previous studies from other coastlines. Pathological findings varied, with one dolphin exhibiting neurobrucellosis-like symptoms and others showing diverse clinical presentations, complicating diagnosis. PCR-based detection indicated a higher infection rate (23.7%), suggesting underestimation in prevalence due to sample preservation status. Multilocus Sequence Typing (MLST) and Multiple-Locus Variable-Number Tandem-Repeat Analysis (MLVA) revealed distinct genetic profiles and close relationships to *B. ceti* strains from the Atlantic, supporting the hypothesis of specific host-adapted lineages in dolphins. These findings underscore the need for further research and surveillance to understand *B. ceti* transmission, host range, and impacts on Atlantic cetaceans, as well as to develop effective diagnostic and management strategies to mitigate infection risks in marine environments.

Keywords: Atlantic Sea; *Brucella ceti*; Cetaceans; Comparative Genomics; Infection surveillance; Marine environment health; Portugal

1. Introduction

Brucella infections of terrestrial mammals have long been recognized and extensively researched; however, it was only during the last years of the twentieth century that the first reports on *Brucella* species from animals living in the marine environment were made, leading later to the inclusion of two novel species in the genus: *Brucella ceti* and *Brucella pinnipedialis* [1]. The first reports on *Brucella*

 doi:10.20944/preprints202412.0676.v1

sp. isolates from marine mammals were from wild harbour seals (*Phoca vitulina*), a harbour porpoise (*Phocoena phocoena*), and a common dolphin (*Delphinus delphi*) in Scotland [2] and a captive bottlenose dolphin (*Tursiops truncatus*) in the USA [3]. *B. ceti* infections have been frequently described in different dolphin species from the Atlantic and the Pacific Oceans [4], as well as from the Mediterranean Sea [5], and it has been recognized as a significant health concern for cetacean populations [6]. These reports have significantly broadened the range of host species known to be affected. In addition, they have extended the area over which the infection is known to occur, to the point where, if serological evidence is included, it seems likely that *Brucella* infection among sea mammals has a global occurrence [2].

B. ceti and *B. pinnipedialis* have been isolated from lungworms and a variety of organs in marine mammals [1,5–9]. In dolphins, *B. ceti* infections can be a challenging diagnosis, as the symptoms are nonspecific and depending on the affected organ or system. This infection is usually associated to meningoencephalomyelitis [1,3,4,7–9], reproductive tract inflammation (orchitis, endometritis, placentitis, endometritis), mastitis, abortion [1,2], discospondylitis, subcutaneous abscesses, and a wide range of other pathological conditions like pneumonia, myocarditis, pericarditis, osteoarthritis, hepatic, splenic, and lymph node necrosis, alongside macrophage infiltration in the liver and spleen, [1–9]. Neurobrucellosis represents a common cause of stranding for cetaceans, being associated with disorientation, uncoordinated lateral swimming, buoyancy disturbances, and death [4,9].

Grattarola and colleagues [10], among different bacteria with a potential zoonotic role identified in cetaceans stranded along the Italian Coastline, *Brucella* spp. were one of the most represented bacterial species, with a prevalence of 4.92%. In fact, there are several reports on *B. ceti* isolation on the Catalonian coast, in Italy, and on the Spanish Mediterranean coast from striped and bottlenose dolphins [9–12]. Additionally, a recent review from Jamil and Colleagues [13] highlights the presence of *B. ceti* and *B. pinnipedialis* infections in different marine mammal species across European regions, including North Atlantic Ocean, the Russian Bering Island, the Croatian Adriatic Sea, the Netherlands, Germany, and Norway. These epidemiological studies are crucial in elucidating the transmission dynamics, prevalence, and risk factors associated with *Brucella* spp. infection in marine mammal populations, not only offering valuable insights into the prevalence and distribution of the pathogen but also informing targeted intervention strategies to help mitigate its spread. Although there are no studies on the prevalence of *Brucella* in wild marine mammals in Portugal, the results above described in surrounding areas, lead us to create the hypothesis that the populations of marine mammals that arrive and pass through Portugal also have the bacteria.

Identifying the etiology of *B. ceti* infections and conducting comprehensive risk assessments are fundamental steps in developing effective biosafety protocols. By characterizing the virulence factors, antibiotic resistance profiles, and transmission routes of *B. ceti*, researchers can assess the potential risks posed to marine mammal populations, aquaculture facilities, and Public Health. *B. ceti* infections in marine mammals can pose a concern for marine conservation efforts [6], as increased pathogen prevalence might interfere with population abundance, by inducing high mortality rates, lowering reproductive success or by synergistically increasing the virulence of other diseases.

In this study, *Brucella* infection in stranded marine mammals in the region of Lisbon and Tagus Valley (Portugal) was investigated to determine prevalence rates, species identification, phylogenetic relationships and perform comparative genomic analysis. We highlight that this is the first time *B. ceti* was isolated from dolphins from the Portuguese coastline.

2. Materials and Methods

2.1. Stranding Data Collection, Necropsy and Tissue Sampling

Post-mortem examinations were performed on 59 marine mammals (*Balaenoptera acutorostrata* n=1; *Delphinus delphis* n=45; *Phoca Vitulina* n=1; *Phocoena phocoena* n=2; *Stenella coeruleoalba* n=6; *Tursiops truncates* n=3; Unidentified n=1) stranded in different beaches along the Lisbon and Tagus Valley coastline, specifically between Lourinhã and Setúbal municipalities, between January 2022 and June 2024. Geographical distribution of the stranding events and species composition of the animals under study is shown in Figure 1. Necropsy and macroscopic evaluation, as well as sample collection, were performed by the Lisbon and Tagus Valley Marine Animals' Stranding Network. Timelapse between receiving the stranding alert and performing necropsy and sample collection was under 12 in all of the investigated animals. A detailed post-mortem examination was performed according to standard

 doi:10.20944/preprints202412.0676.v1

protocols [14], and sample collection depended on the carcasses' preservation status. Individual data, including sex, decomposition code and nutritional condition, along with stranding data concerning the geographical distribution, and date of occurrence, were registered. During necropsies, samples from tissues (brain, spleen, liver, lung, mammary gland, testis/uterus, and several limph nodes), vaginal and preputial swabs, and/or fluids (blood, milk) were collected, and kept frozen at <-16ºC until tested. All samples (Supplementary file Table S1) were submitted to the Animal Health National Reference Laboratory for microbiological and molecular investigations focused on Brucella infection diagnosis (INIAV, Oeiras, Portugal).

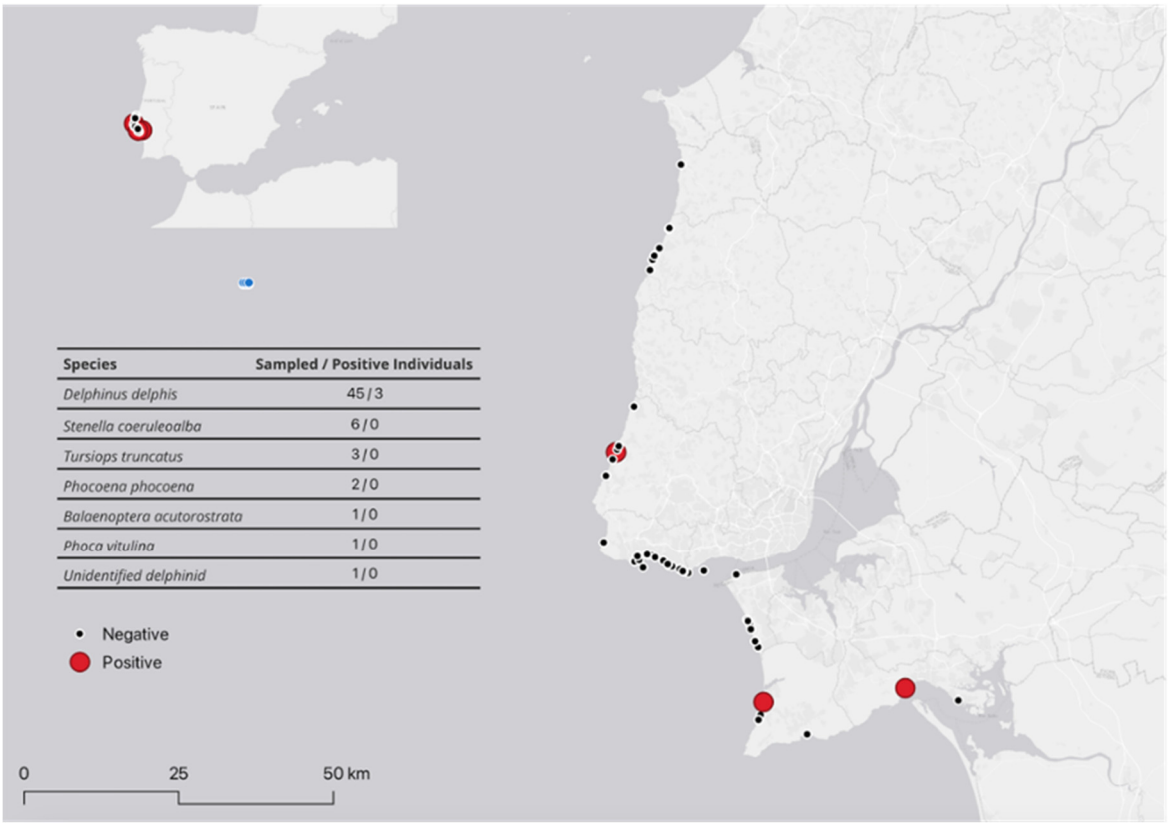

Figure 1. Geographical distribution of the stranding events and species composition of the animals under study.

2.2. Brucella Isolation and Identification

The primary isolation of *Brucella* spp. was performed on all tissues, swabs, and fluids available from the 59 animals stranded on different beaches along the Lisbon Coastline between 2022 and June 2024. Samples were homogenized under sterile conditions in the minimum possible amount of sterile buffered saline (PBS pH 6.8) in a Stomacher unit (Seward Medical, Worthing, UK), and 0.2 mL of each tissue homogenate inoculated on two plates of both Farrell's [15] and CITA [16] selective media. The plates were observed for microbial growth following 5–10 days of incubation at 37°C in both ambient air and a 5% CO_2 environment. A culture was considered positive when at least one *Brucella* colony forming unit (CFU) was isolated. Suspected colonies were further identified and characterized by standard bacteriological procedures, based on CO_2 requirement, H_2S production, oxidase test, urea hydrolysis, agglutination with monospecific sera anti-A, anti-M, and anti-R, and fuchsin and thionine dye sensitivity [17].

2.3. Molecular Methods

Bacterial genomic DNA extraction from available tissues was performed in a nucleic acid extraction workstation, Kingfisher Flex (Thermo Fisher Scientific, Waltham, MA, USA), using the IndiMag Kit (Indical Bioscience, Leipzig, Germany), following the manufacturer's instructions. After extraction, the DNA was stored at 4° C. All samples were tested using RT-PCR targeting *bcsp*31 and *per* genes, as described previously [18], for identification of *Brucella* spp. Briefly, real-time TaqMan PCR was set up in a final volume of 25 µl, 1x TaqMan Universal PCR Master Mix (Applied

 doi:10.20944/preprints202412.0676.v1

Biosystems, France), each primer and TaqMan probe at concentrations of 0.3 µM and 0.25 µM, respectively, and 3 ng of DNA template. The reaction mixture was initially incubated for 10 min at 95° C. Amplification was performed for 45 cycles of denaturation at 95° C for 15 s, annealing and extension at 60 ° C for 1 min. The PCR reaction was performed on a Bio-Rad CFX Maestro 2.3 (Bio-Rad, France). A result was considered positive when an amplification curve with a Ct value less than 38 was obtained for both targets.

Genomic DNA from each *Brucella spp.* isolated from this work, and control strains, were extracted with PureLink Genomic DNA Mini Kit (Invitrogen, USA), stored at -20° C until used. Brucellae isolates were identified using the multiplex PCR Bruce-ladder as described elsewhere [19]. DNA samples were also tested by Multiple loci variable number of tandem repeats analysis (MLVA-16) as previously described [19]. The 16 *loci* have been classified in three panels, named panel 1, composed of 8 minisatellite (bruce06, bruce08, bruce11, bruce12, bruce42, bruce43, bruce45 and bruce55), panel 2A (bruce18, bruce19 and bruce21) and panel 2B (bruce04, bruce07, bruce09, bruce16 and bruce30) composed of three and five microsatellite markers, respectively. Briefly, PCR reactions were performed in a total volume of 15 µl containing 3 ng of DNA, 1 × PCR Reaction Buffer, 1 U of Taq DNA polymerase (Promega, USA), 200 µM of each dNTPs and 0.3 µM of each flanking primers. An initial denaturation step at 96° C for 5 min was followed by 30 cycles of denaturation at 96° C for 30 s, primer annealing at 60° C for 30 s and elongation at 70° C for 1 min. The final extension step was performed at 70° C for 5 min. Amplification products were loaded on a 3% standard agarose gel to analyze panel 2A and 2B *loci* (tandem repeats with a unit length shorter than 8 bp), and on a 2% standard agarose gel for panel 1 *loci* (tandem repeats with a unit length larger than 10 bp), with suitable molecular size markers. The total number of repeats at each locus was determined by the correlation with the amplicon size according to the 2013 *Brucella* allele assignment table (Le Flèche et al., 2006 version 3.6 available at http:// mlva.u-psud.fr). Genomic DNA from *B. melitensis* biovar 1 strain 16 M (ATCC 23456) and *B. ceti* Atlantic dolphin type (B14/94) were used as controls for alleles assignment.

2.4. Whole Genome Sequencing and Bioinformatic Analysis

Whole Genome Sequencing (WGS) was used to evaluate the genetic structure of *B. ceti* isolates to increase knowledge into brucellae genome evolution. The extracted nucleic acids were assessed for quality and quantity using spectrophotometry and dsDNA specific fluorescence-based assays. WGS was performed as previously described [21,22]. Briefly, to recover the genomic structure of these isolates as accurately as possible, the extracted long strand high-grade DNA was directly sequenced using native long-read Nanopore sequencing (GridION X5 sequencing platform, Oxford Nanopore Technologies) at a minimum coverage depth of 200X, using Nanopore V14 kit chemistry with R10.4.1 pore. The sequencing data was analyzed with a customized pipeline developed by BioISI Genomics for genome assembly. The analysis included base-calling, pre-filtering based on read size and quality, and subsequent assembly, which involved the identification of bacterial species using Kraken, plasmid identification with PlasmidFinder [23], clonal origin analysis through phylogenetic methods with ParSNP [24], pathogenicity prediction using PathogenFinder [25], identification of acquired virulence genes via VirulenceFinder and VFDB [26], identification of antibiotic resistance genes with CARD [27], MegaRES [28], and ResFinder [29], as well as Multi-Locus Sequence Typing (MLST) [30,31] and Multiple Locus Variable-number Tandem Repeat Analysis (MLVA) [32].

For bioinformatic analysis, only reads with a Q score > 7 (minimum value from MinKNOW) were retained. Duplex base calling was performed using Dorado (v0.3.1+bb8c5ee) with the dna_r10.4.1_e8.2_400bps_sup@v4.2.0 model. Assembly was conducted with Canu (v2.0) [33] using parameters: genomeSize = 3.4M and -nanopore-raw. A quality report for the assemblies was generated using Quast (v5.0.2) with parameters set for a prokaryotic genome assembly. The Canu assemblies served as input for various bioinformatic tools available at https://genomicepidemiology.org/services/: Abricate v1.0.1 with VFDB [25,33], CARD, MegaRES [27,28], and PlasmidFinder (v2.1, “Gram Negative” database option) [25]. The assemblies were also analyzed using MLST tool (v2.0, with the configuration specific to *Brucella spp.*) and MLVA bank for Microbes genotyping (v1.4.0, configured for *Brucella* v4_6_5).

3. Results

 doi:10.20944/preprints202412.0676.v1

3.1. Brucella Infection Diagnosis and Brucella Isolates Characterization

The isolation of *Brucella* spp. suspect colonies was obtained in three common dolphins (*Delphinus delphis*) out of the 59 animals investigated (5.1%). Suspected colonies were isolated from brain in DDE001 and DDE003 dolphins, and from spleen, liver, lung and lymph nodes in all the three animals. All isolates were typed by bacteriological and molecular methods and assigned as *B. ceti*. The phenotypic features regarding CO_2 requirement, H_2S production, oxidase and urea tests, agglutination with monospecific sera, and fuchsin and thionine sensitivity, were consistent with previously described *B. ceti* patterns: none of the three isolates required CO_2 for growth or produced H_2S, but all presented smooth phenotype, were oxidase and urease positive, agglutinate with anti-A monospecific serum, and grew in the presence of thionine and fuchsin dyes. Additionally, *Brucella* spp. DNA was detected in tissues from 14 animals (14/59; 23.7%), including the three dolphins with *B. ceti* isolation, and one *Stenella coeruleoalba,* and one *Foca vitulina*. The majority of the positive results were attained from the brain, spleen, liver, lung, lymph nodes, uterus and/or testis. In one suspected animal, DDE040, it was not possible to obtain a pure culture of the isolate, therefore phenotypic tests were inconclusive. However, the PCR carried out on the DNA extracted from the brain sample showed a positive result. *B. ceti*, isolates were also characterized by the multiplex Bruce-ladder multiplex, and all presented the molecular pattern comprising two fragments of 774 bp and 550 bp. Lastly, MLVA-16 analysis was performed and data compared to results obtained by other authors with *B. ceti* strains from different origins, which were deposited in the *Brucella* MLVA database (available at http://mlva.u-psud.frhttp://mlva.u-psud.fr). In this work, MLVA-11 (panels 1 and 2A markers) discriminated 2 genotypes (GT), dividing *B. ceti* DDE001 into GT8, and DDE002 and DDE003 isolates into GT-17. The results obtained during this study are summarized in Table 1, and in the Supplementary file Table S1.

Table 1. Confirmed and suspected positive animals, and respective qPCR and bacteriological results.

Animal Identification[1]	Sex/Age[2]	Status[3]	Region	Year of stranding	qPCR positive samples [4]	Bacteriological suspected samples	*Brucella* characterization
DDE001	F/J	2	Sintra	2022	B,L,Lg,LN,S	B,L,Lg,S	*B. ceti*
DDE002	M/J	2	Setúbal	2023	B,L,Lg,LN,S	L,Lg,LN,S	*B. ceti*
DDE003	F/A	1	Sesimbra	2023	B,C	B,L,Lg,LN,S	*B. ceti*
DDE005	F/A	4	Setúbal	2022	B,L,Lg,S	N	N
DDE008	M/A	2	Sesimbra	2022	B,L,Lg,S	N	N
DDE013	M/J	2	Cascais	2022	LN	N	N
SCO016	M/A	2	Almada	2023	B	N	N
DDE027	M/J	2	Cascais	2023	B,L,Lg,LN,S,T	N	N
DDE033	F/A	1	Cascais	2023	LN	N	N
PVI035	M/J	4	Cascais	2023	T	N	N
DDE036	M/A	4	Cascais	2023	LN	N	N
DDE037	F/A	4	Cascais	2023	LN	N	N
DDE040	M/J	2	Cascais	2023	B	B	Inconclusive
DDE043	M/J	2	Cascais	2023	PS	N	N

[1] DDE: *Delphinus delphis*; PVI: *Phoca vitulina*; SCO: *Stenella coeruleoalba.* [2] A: adult; F: female; J: juvenil; M: male. [3] Carcasses'preservation status: 1 (just died), 2 (fresh carcass), 3 (moderate decomposition), 4 (advanced decomposition). [4] B: brain; C: cervix; L: liver; Lg: lung; LN: limph nodes; N: negative; PS: prepucial swab; S: spleen; T: testis.

3.2. Stranding Data and Necropsy Findings from B. ceti Positive Animals

Although post-mortem investigations were carried out on the 59 stranded animals, we will only focus on the observations of the three bacteriologically positive animals. Dolphins DDE001and DDE002 were juveniles, while DDE003 was an adult. DDE001 was a female dolphin that stranded in March 2022 in Sintra, Portugal (38.8545207, -9.4542235). The animal presented moderate nutritional condition and a physical exam revealed skin lacerations and traumatic lesions consistent with

 doi:10.20944/preprints202412.0676.v1

bycatch. Macroscopic evaluation of internal organs disclosed moderate pulmonary edema and the presence of gas embolism in the renal, mesenteric and thoracic vasculature. Additionally, mild splenic hyperplasia, with the presence of dark colored papules (0.5 cm diameter) at the organ's surface and decreased consistency, was also observed. DDE002 was a male dolphin that stranded in March 2023 in Setúbal, Portugal (38.5094191, -8.9217497). The presence of live ectoparasites suggested a recent death. The animal was in a fair nutritional condition and presented a linear single deep laceration (1.4 cm depth, until the muscular layer) in one side of the body, suggesting a traumatic etiology. Pathological findings included a generalized lung hyperemia and a single ulcerative lesion in the oral cavity (2 cm diameter). DDE003 was a female dolphin that live stranded in July 2023 in Sesimbra, Portugal (38.4887533, -9.1840278). Reports indicate that the animal was disoriented and unable to swim and keep floatability alone. Despite efforts from people present on the beach to refloat the animal, it died shortly. The animal was severely emaciated. Pathological findings included moderate splenic hyperplasia, generalized lung hyperemia with associated nematode parasitic infection, the presence of petechiae in the intestinal mucosa, and congested areas in the meninges and the internal side of the skull.

3.3. Comparative genomic analysis and phylogenetic relationship of B. ceti isolates

The characterization of the isolates from dolphins DDE001, DDE002, and DDE003 by whole-genome sequencing, at a minimum coverage depth of 200X, confirmed them as members of *B. ceti* species. The key findings are summarized in Table 2.

Table 2. Summary of the main genomic results for dolphins *B. ceti* isolates.

***B. ceti* isolates**	**DDE001**	**DDE002**	**DDE003**
B. ceti strains M13/05/1 alignment	3077 SNVs, 279 insertions, 257 deletions	2956 SNVs, 248 insertions, 241 deletions	2478 SNVs, 212 insertions, 215 deletions
B. ceti strain M644/93/1 alignment	5855 SNVs, 505 insertions, 562 deletions	4996 SNVs, 439 insertions 457 deletions	4997 SNVs, 439 insertions, 464 deletions
Virulence genes	*bspE, vceA, btpB, manAoAg, wbkC, virB7, lpxA, lpxK, manCcore*		
Antibiotic resistance genes	*mprF, bepC* and *D, E, F, G*		
MLST [1]	ST-49		ST-26
cgMLST [2]	cgST-392		cgST-340
MLVA [3]	Cluster A1		Cluster A2

[1] MLST: Multlocus sequence typing (ST: Sequence Type from the typing database https://pubmlst.org/ for the species *Brucella* spp.);. [2] cgMLST: core genome MLST (cgST from the typing database https://pubmlst.org/ for the species *Brucella* spp.);. [3] MLVA: Multiple-Locus Variable-Number Tandem Repeat Analysis; cluster attribution based on: Maquart *et al.* 2009 [32].

The ParSNP analysis used the 10 assembled contigs of *B. ceti* strains available on NCBI as of February 2024 (https://blast.ncbi.nlm.nih.gov/) (see Supplementary file Table S2). This analysis revealed that isolates DDE002 and DDE003 are more closely related to each other than to DDE001. Additionally, strains M13/05/1 and M644/93/1 were found to have the closest relationship with all three isolates. Alignments were performed between each isolate and the closely related *B. ceti* strains M13/05/1 and M644/93/1, as well as among the Portuguese isolates, to evaluate single nucleotide variations (SNVs), insertions, and deletions. This analysis allowed for the construction of a phylogenetic tree. Detailed information on the variations in SNVs, insertions, and deletions in comparison to the closely related *B. ceti* strains and among the Portuguese isolates is summarized in Tables 2 and 3, respectively, with the resulting phylogenetic tree illustrated in Figure 2. VirulenceFinder identified acquired virulence genes in *Brucella* isolates, as detailed in Supplementary file Table S3. For 100% identity and coverage, virulence factors were consistent across all isolates. However, in the core gene *manCcore*, isolates DDE002 and DDE003 showed a 99% identity, indicating a single mismatch when compared to *B. ceti* strain M644/93/1, while DDE001 had a 100% match. The

 doi:10.20944/preprints202412.0676.v1

identified virulence genes across the isolates were *bspE*, *vceA*, *btpB*, *manAoAg*, *wbkC*, *virB7*, *lpxK*, *lpxA*, and *manCcore*. The ABRicate pipeline, using CARD and MegaRES databases, identified six acquired antibiotic resistance genes (ARGs): *mprF*, which is commonly linked to resistance to cationic antimicrobial peptides due to its role in modifying cell membrane charge, and genes *bepC, D, E, F* and *bepG*, generally associated with resistance mechanisms that may include antibiotic modification or efflux (Supplementary file Table S4).

Table 3. Single Nucleotide Variants (SNVs), insertions and deletions within the Portuguese isolates.

***B. ceti* isolates**	**DDE001**	**DDE002**	**DDE003**
DDE001	----------	213 SNVs, 12 insertions, 18 deletions	216 SNVs, 16 insertions, 20 deletions
DDE002	213 SNVs, 12 insertions, 18 deletions	----------	107 SNVs, 13 insertions, 11 deletions
DDE003	216 SNVs, 16 insertions, 20 deletions	107 SNVs, 13 insertions, 11 deletions	----------

MLST and MLVA profiles (Table 2, and Supplementary file Table S5), revealed that DDE001 aligned with Sequence Type 49 (ST-49) profile (cg-ST392), while isolates DDE002 and DDE003 matched ST-26 profile (cg-ST340). The difference between ST-49 and ST-26 lies at the *cobQ* locus, where a single nucleotide polymorphism (SNP) occurs at position 213 bp within the 423-bp gene. This SNP is marked by a 'C' (allele 6) in ST-49 and a 'T' (allele 10) in ST-26. Additionally, MLVA profiling showed that isolate DDE001 exhibited close similarity to *B. ceti* strains M57/07/1 and M260/03/1, which were isolated from the spleen and brain, respectively, of Atlantic white-sided dolphins (*Lagenorhynchus acutus*) in Scotland, with both strains falling under the A1 cluster [32]. Isolate DDE002 was closely related to *B. ceti* strain M654/99/1 from a striped dolphin's brain, also in Scotland, and classified within the A2 cluster [32]. Meanwhile, DDE003 showed close resemblance to *B. ceti* strains M654/99/1, M83/07/1, M231/07/3, M267/05/4, M83/07/3, and M267/05/1, which were obtained from the brain, kidney, and colorectal lymph nodes of Striped (*Stenella coeruleoalba)*, Atlantic White-Sided, and bottlenose dolphins, all belonging to the A2 cluster [32].

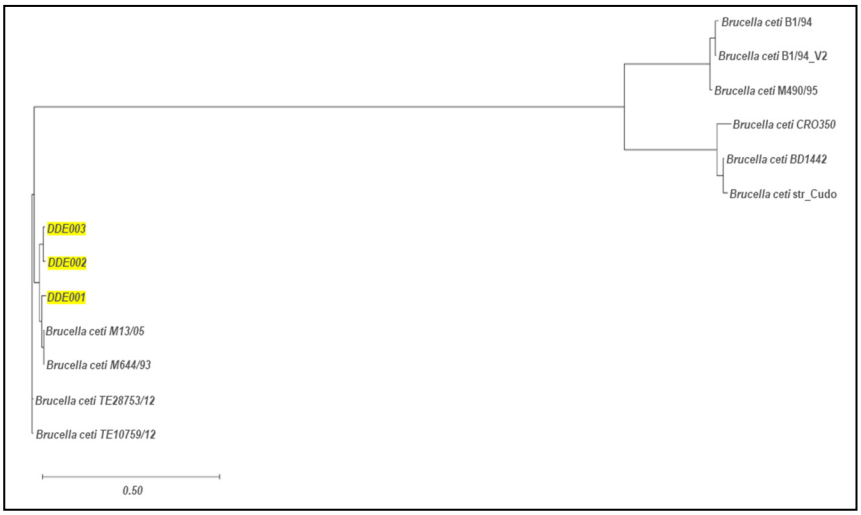

Figure 2. Phylogenetic tree generated by ParSNP. The phylogenetic tree was based on 689 SNVs and is rooted with *Brucella ceti* TE28753-12. The branch length is proportional to the number of SNVs (the scale bar represents the difference in SNVs). The strains from this study are marked with a yellow square.

4. Discussion

 doi:10.20944/preprints202412.0676.v1

This study investigates Brucella infection in marine mammals stranded along the Lisbon and Tagus Valley coast from 2022 to mid-2024. In out of the 59 marine mammals examined, *B. ceti* was isolated in three common dolphins (5.1%). This finding highlight, for the first time, the presence of *Brucella* in marine mammals in Portugal coastline, expanding the dispersion area of this bacteria. The observed prevalence (5.1%) is like the 4.92% prevalence obtained by Grattarola and colleagues [10] in cetaceans stranded along the Italian Coastline.

As referred previously, *B. ceti* has been associated with a range of pathological changes in cetaceans. In this study, dolphins in which *B. ceti* isolation was achieved exhibited distinct pathological findings, with some being coincident with the ones expected for *Brucella* infections. For instance, dolphin DDE003, with isolation of *B. ceti* and *Brucella* DNA detection on the central nervous system, presented meningitis, associated to disorientation and inability to swim and maintain equilibrium and flotation, suggesting a situation of neurobrucellosis, like the findings in a dolphin of the Mediterranean Catalonian coast [4]. It also displayed severe emaciation, parasitic infection, and multiple signs of systemic distress, including splenic hyperplasia. DDE001 and DDE002 presented pathological changes typically not consistent with *B. ceti* infection, underlying the diverse clinical presentations of *Brucella* infections, already referred by other authors, and complicating the diagnosis in marine mammals. Furthermore, it is interesting to highlight that the three cases display different likely death causes, reinforcing the idea that susceptibility to this pathogen can differ between individuals and be linked to different ecological drivers.

Although *B. ceti* isolation was achieved in 5,1% (3/59) of dolphins the results obtained by PCR for *Brucella* spp. detection directly from the same tissues were higher, with molecular confirmation in 14 animals (23.7%), including those three *Brucella*-positive, suggesting that the incidence of this agent might be higher in these species. In fact, the three isolates here identified were obtained in samples from animals with code 1 (recently died) or 2 (fresh carcass) of preservation status [14], highlighting that *Brucella* isolation success may decrease with increased time between death and sampling.

The MLST analysis revealed distinct genetic profiles, indicating diversity within *B. ceti* strains. ST-26 was attributed to the strains from animals DDE002 and DDE003, also previously identified in isolates from dolphins of the Mediterranean Sea and North America. This sequence type is exclusively observed in dolphin isolates, forming the cluster A [4,5,8]. The isolate from DDE001 was assigned to ST-49, which was also observed in 17 *B. ceti* isolates, two in Spain and the remaining in Scotland, between 2006 and 2019 (PubMLST, available online: https://pubmlst.org/organisms?title=Brucella+spp. Accessed on 15/11/2024). Additionally, MLVA showed a close relation between the isolates from this work and isolates from the Atlantic Sea, although DDE001 falling under the A1 cluster, and DDE002 and DDE003 falling under A2 cluster [32]. Up to now, all *B. ceti* Mediterranean strains stem in a separate branch from the main MLVA A1 and A2 clusters of *B. ceti* isolates from dolphins inhabiting the Atlantic Ocean [4,5]. This close MLVA similarity to other dolphin-derived strains supports the hypothesis of specific host-adapted *Brucella* lineages in marine environments [35]. Also, the comparative genomic analysis showed a close relationship with other *B. ceti* strains previously isolated from dolphins, namely those obtained from dolphins in the Scottish sea [32], indicating possible common sources or transmission pathways within marine environments. In fact, the phylogenetic analysis based on the SNVs revealed that isolates DDE002 and DDE003 are more closely related to each other than to DDE001, belonging respectively to the cluster A2 and A1, suggesting potential differences in infection sources or transmission routes. Virulence genes were consistent across the isolates, reinforcing the pathogenic potential of *B. ceti*, with minor variations in the *manCcore* gene suggesting slight genetic diversity. Eight virulence associated genes were identified in the *B. ceti* isolates: *bspE* gene, associated with pathogenesis, and likely aids in host interaction, enhancing *B. ceti*'s ability to cause disease; *vce*A gene, part of the *virB* operon, is essential for the Type IV secretion system, crucial for the intracellular survival as it allows *Brucella* to manipulate host cells; *btpB* gene is involved in host interactions and the modulation of the host immune response, probably contributing to the ability of *B. ceti* to evade the host immune system; *manAoAg* gene, involved in the biosynthesis of mannose-containing O-antigen; *wbkC* gene, associated with O-polysaccharide biosynthesis, which contributes to the structure of the bacterial cell surface; *virB7* gene, also part of the Type IV secretion system, which is crucial for intracellular survival and virulence as this system allows *Brucella* to translocate effector

 doi:10.20944/preprints202412.0676.v1

molecules into host cell; *lpxK* and *lpxA* genes, involved in lipid A biosynthesis; and *manCcore* gene (required for synthetizing LPS core oligosaccharide), which is vital to *Brucella*'s outer membrane and pathogenicity. Furthermore, the analysis performed using the CARD and MegaRES databases highlighted the presence of six genes potentially involved in AMR in the three *B. ceti* genomes: the multiple peptide resistance factors *mprF*, and the outer membrane efflux proteins *bep C, D, E, F*, and *G*. These results are in accordance with other authors that in a recent study, investigated and compared a panel of *B. ceti* and *B. pinnipedialis* genome sequences [36]. The identification of six antibiotic resistance genes (ARGs) in these isolates raises concerns regarding treatment challenges, highlighting the importance of monitoring ARGs in *B. ceti* to better understand and manage the risks associated with resistance in marine mammal pathogens.

5. Conclusions

In conclusion, the demonstration of the existence of *B. ceti* in dolphins for the first time in Portugal, highlights the need of further studies with greater geographic coverage to assess the infection's true prevalence along this coastline. These findings shed light on the occurrence, and characteristics of *B. ceti* infection in dolphins, and emphasize the need for continuous surveillance and advanced diagnostic methods to better understand and manage *Brucella* infections in marine mammals. Continued research on *B. ceti* infection is crucial to further understand its transmission dynamics, host range, and its impact on cetacean populations in the Atlantic Sea. Additionally, there is a need for the development of effective diagnostic tools and sustainable management strategies to mitigate the spread of this infection and preserve the health of affected cetaceans.

Supplementary Materials: Table S1: Cetacean strandings along the Lisbon and Tagus Valley Coastline (January 2022 – June 2024): Animal details, Sample collection, and Bacteriological and Molecular results. Table S2: Assembled *Brucella ceti* contigs from NCBI utilized in ParSNP analysis. Table S3: Identification of acquired virulence genes. Table S4: Identification of acquired antibiotic resistance genes. Table S5: *Brucella ceti* Multi-Locus Sequence Typing (MLST) and Multiple-Locus Variable Number Tandem-Repeat Analysis (MLVA) alleles profiles and clustering.

Author Contributions: Conceptualization, S.C., M.L.G., R.D., and A.C.F.; Formal analysis S.C, M.L.G., M.N., P.P., M.P., R.D., and A.C.F.; Investigation, S.C., M.L.G., M.N., P.P., M.P., C.F., A.B.C., S.P., R.D. and. A.C.F.; Methodology, S.C., M.L.G, M.N., P.P., M.P., R.D., and A.C.F.; Writing—original draft preparation, S.C., and A.C.F.; Writing—review and editing, S.C., M.L.G, M.N., R.D., and A.C.F. All authors have read and agreed to the published version of the manuscript.

Funding: Not applicable.

Institutional Review Board Statement: The study protocol was approved by the National Competent Authority (ICNF,), under permits: LICENÇA Nº 170 / 2022 / CAPT, LICENÇA Nº 171 / 2022 / CAPT, LICENÇA Nº 883 / 2022 / CAPT, LICENÇA Nº 464 / 2023 / CAPT, LICENÇA Nº 465 / 2023 / CAPT, LICENÇA Nº 463 / 2023 / CAPT, LICENÇA Nº 462 / 2023 / CAPT, LICENÇA Nº 011 / 2024 / CAPT, LICENÇA Nº 014 / 2024 / CAPT, LICENÇA Nº 012 / 2024 / CAPT, LICENÇA Nº 009 / 2024 / CAPT, LICENÇA Nº 013 / 2024 / CAPT, LICENÇA Nº 010 / 2024 / CAPT.

Informed Consent Statement: Not applicable.

Data Availability Statement: The original contributions presented in this study are included in the article. The WGS data is submitted at NCBI GenBank with the following accession number: SAMN45131997, SAMN45131998, and SAMN45131999 for DDE001, DDE002 and DDE003 genome sequences, respectively.

Acknowledgments: Work supported by UIDB/04046/2020 and UIDP/04046/2020 Centre grants from FCT, Portugal (to BioISI).

Conflicts of Interest: The authors declare no conflicts of interest.

 doi:10.20944/preprints202412.0676.v1

References

1. Foster G., Osterman B. S., Godfroid J., Jacques I., Cloeckaert A. *Brucella ceti* sp. Nov. and *Brucella pinnipedialis* sp. nov. for *Brucella* strains with cetaceans and seals as their preferred hosts. *Int. J. Syst. Evol. Microbiol*. **2007**, 57, 2688–2693 10.1099/ijs.0.65269-0 Ross et al., 1994.
2. Foster G., MacMillan A.P., Godfroid J., Howie F., Ross H.M., Cloeckaert A., Reid R. J., Brew S., Patterson I. A. A review of *Brucella* spp. infection of sea mammals with particular emphasis on isolates from Scotland. *Vet. Microbiol*. **2002**, 90, 563–580 10.1016/S0378-1135(02)00236-5
3. Ewalt D.R., Payeur J. B., Martin B. M., Cummins D.R., Miller G. M. Characteristics of a *Brucella* species from a bottlenose dolphin (*Tursiops truncates*). *J. Vet. Diagn. Invest*. **1994**, 6, 448–452 10.1177/104063879400600408
4. Isidoro-Ayza, M., Ruiz-Villalobos, N., Pérez, L., Guzmán-Verri, C., Muñoz, P.M., Alegre, F., Barberán, M., Chacón-Díaz, C., Chaves-Olarte, E., González-Barrientos, R., Moreno, E., Blasco, J.M., Domingo, M. *Brucella ceti* infection in dolphins from the Western Mediterranean sea. *BMC Vet. Res*. **2014**, 10:206. Doi: 10.1186/s12917-014-0206-7.
5. Garofolo, G., Petrella, A., Lucifora, G., Di Francesco, G., Di Guardo, G., Pautasso, A., Iulini, B., Varello, K., Giorda, F., Goria, M., *et al*. Occurrence of *Brucella ceti* in Striped Dolphins from Italian Seas. *PloS ONE 15*, **2020**, e0240178.
6. Guzmán-Verri, C., González-Barrientos, R., Hernández-Mora, G., Morales, J.Á., Baquero-Calvo, E., Chaves-Olarte, E., Moreno, E. *Brucella ceti* and brucellosis in cetaceans. *Front Cell Infect Microbiol*. **2012**, Doi: 10.3389/fcimb.2012.00003. PMID: 22919595; PMCID: PMC3417395.
7. Nymo, I.H., Tryland, M., Godfroid, J. A Review of *Brucella* Infection in Marine Mammals, with Special Emphasis on *Brucella pinnipedialis* in the Hooded Seal (Cystophora cristata). *Vet. Res*. **2011**, 42, 93.
8. Whatmore, A.M., Dawson, C., Muchowski, J., Perrett, L.L., Stubberfield, E., Koylass, M., Foster, G., Davison, N.J., Quance, C., Sidor, I.F., *et al*. Characterisation of North American *Brucella* Isolates from Marine Mammals. *PloS ONE*, **2017**, 12, e0184758.
9. Grattarola, C., Petrella, A., Lucifora, G., Di Francesco, G., Di Nocera, F., Pintore, A., Cocumelli, C., Terracciano, G., Battisti, A., Di Renzo, L., *et al*. *Brucella ceti* Infection in Striped Dolphins from Italian Seas: Associated Lesions and Epidemiological Data. *Pathogens* **2023,** 12, 1034. https://doi.org/10.3390/pathogens12081034.
10. Grattarola, C., Pietroluongo, G., Belluscio, D., Berio, E., Canonico, C, Centelleghe, C., Cocumelli, C., Crotti, S., Denurra, D., Di Donato, A., *et al*. Pathogen Prevalence in Cetaceans Stranded along the Italian Coastline between 2015 and 2020. Pathogens 2024, 13, 762. https://doi.org/10.3390/pathogens13090762.
11. Alba, P., Terracciano, G., Franco, A., Lorenzetti, S., Cocumelli, C., Fichi, G., Eleni, C., Zygmunt, M.S., Cloeckaert, A., Battisti, A. The presence of *Brucella ceti* ST26 in a striped dolphin (*Stenella coeruleoalba*) with meningoencephalitis from the Mediterranean Sea. Vet Microbiol. 2013 May 31;164(1-2):158-63. doi: 10.1016/j.vetmic.2013.01.023. Epub 2013 Feb 1. PMID: 23419820.
12. Isidoro-Ayza, M., Ruiz-Villalobos, N., Pérez, L., Guzmán-Verri, C., Muñoz, P.M., Alegre, F., Barberán, M., Chacón-Díaz, C., Chaves-Olarte, E., González-Barrientos, R., Moreno, E., Blasco, J.M., Domingo, M. *Brucella ceti* infection in dolphins from the Western Mediterranean sea. BMC Vet Res 10, 206 (2014). https://doi.org/10.1186/s12917-014-0206-7.
13. Jamil, T., Akar, K., Erdenlig, S., Murugaiyan, J., Sandalakis, V., Boukouvala, E., Psaroulaki, A., Melzer, F., Neubauer, H., Wareth, G. Spatio-Temporal Distribution of Brucellosis in European Terrestrial and Marine Wildlife Species and Its Regional Implications. Microorganisms 2022, 10, 1970. https://doi.org/10.3390/microorganisms10101970.
14. IJsseldijk, L.L., Brownlow, A.C. & Mazzariol, S. Best practice on cetacean post mortem investigation and tissue sampling – Joint ACCOBAMS and ASCOBANS. document.https://www.ascobans.org/sites/default/files/document/ascobans_ac25_inf3.2_rev1_best-practice-cetacean-post-mortem-investigation.pdf [Accessed August 3, **2023**].
15. Farrell, I.D. The development of a new selective medium for the isolation of *Brucella abortus* from contaminated sources. *Res Vet Sci*, **1974,** 16:280–286.
16. De Miguel, M.J., Marín, C.M., Muñoz, P.M., Dieste, L., Grilló, M.J., Blasco, J.M. Development of a selective culture medium for primary isolation of the main *Brucella* species. *J Clin Microbiol*, **2011,** 49:1458–1463.
17. World Organization for Animal Health. Brucellosis (infection with *B. abortus, B. melitensis* and *B. suis*). OIE Terrestrial Manual 2022.
18. Bounaadja, L., Albert, D., Chénais, B., Hénault, S., Zygmunt, M.S., Poliak, S., Garin-Bastuji , B. Real-time PCR for identification of *Brucella* spp.: a comparative study of *IS*711, *bcsp*31 and *per* target genes. *Vet Microbiol.* **2009,** 28;137(1-2):156-64. doi: 10.1016/j.vetmic.2008.12.023. Epub 2009 Jan 4. PMID: 19200666.
19. Ferreira, A.C., Corrêa de Sá, M.I., Dias, R. and Tenreiro, R. MLVA-16 typing of *Brucella suis* biovar 2 strains circulating in Europe. *Vet Microbiol.* **2017,** 210, pp. 77-82. doi: 10.1016/j.vetmic.2017.09.001.
20. López-Goñi, I., García-Yoldi, D., Marín, C.M., de Miguel, M.J., Barquero-Calvo, E., Guzmán-Verri, C., Albert, D., Garin-Bastuji, B. New Bruce-ladder multiplex PCR assay for the biovar typing of *Brucella suis* and the discrimination of *Brucella suis* and *Brucella canis*. *Vet. Microbiol*. **2011,** 154, 152–155.

21. Molina-Mora, J. A., Campos-Sánchez, R., Rodríguez, C., Shi, L., & García, F. High quality 3C de novo assembly and annotation of a multidrug resistant ST-111 Pseudomonas aeruginosa genome: Benchmark of hybrid and non-hybrid assemblers. *Scientific reports*, **2020,** 10(1), 1392. https://doi.org/10.1038/s41598-020-58319-6.
22. Ruan, Z., Wu, J., Chen, H., Draz, M. S., Xu, J., & He, F. Hybrid Genome Assembly and Annotation of a Pandrug-Resistant Klebsiella pneumoniae Strain Using Nanopore and Illumina Sequencing. *Infection and drug resistance*, **2020,** 13, 199–206. https://doi.org/10.2147/IDR.S240404.
23. Carattoli, A., Hasman, H. PlasmidFinder and In Silico pMLST: Identification and Typing of Plasmid Replicons in Whole-Genome Sequencing (WGS). In: de la Cruz, F. (eds) Horizontal Gene Transfer. *Methods in Molecular Biology*, **2020**, vol 2075. Humana, New York, NY. https://doi.org/10.1007/978-1-4939-9877-7_20.
24. Kille, B., Nute, M.G., Huang, V., Kim, E., Phillippy, A.M., Treangen, T.J. Parsnp 2.0: Scalable Core-Genome Alignment for Massive Microbial Datasets. *bioRxiv*. **2024**doi: https://doi.org/10.1101/2024.01.30.577458
25. Cosentino S, Voldby Larsen M, Møller Aarestrup F, Lund O. PathogenFinder--distinguishing friend from foe using bacterial whole genome sequence data. *PLoS One*. **2013,** 8(10):e77302. doi: 10.1371/journal.pone.0077302. Erratum in: *PLoS One*. 2013;8(12). doi:10.1371/annotation/b84e1af7-c127-45c3-be22-76abd977600f. PMID: 24204795; PMCID: PMC3810466.
26. Chen, L. VFDB: a reference database for bacterial virulence factors. *Nucleic Acids Research*, **2004,** 33(Database issue), D325–D328. https://doi.org/10.1093/nar/gki008
27. Alcock, B.P., Huynh, W., Chalil, R., Smith, K.W., Raphenya, A.R., Wlodarski, M.A., Edalatmand, A., Petkau, A., Syed, S.A., Tsang, K.K., *et al.* CARD 2023: expanded curation, support for machine learning, and resistome prediction at the Comprehensive Antibiotic Resistance Database. *Nucleic Acids Res*. **2023,** 51(D1):D690-D699. doi: 10.1093/nar/gkac920. PMID: 36263822; PMCID: PMC9825576.
28. Lakin, S.M., Dean, C., Noyes, N.R., Dettenwanger, A., Ross, A.S., Doster, E., Rovira, P., Abdo, Z., Jones, K.L., Ruiz, J., Belk, K.E., Morley, P.S., Boucher, C. MEGARes: an antimicrobial resistance database for high throughput sequencing. *Nucleic Acids Res*. **2017,** 45(D1):D574-D580. doi: 10.1093/nar/gkw1009.
29. Larsen, M. V., Cosentino, S., Lukjancenko, O., Saputra, D., Rasmussen, S., Hasman, H., Sicheritz -Pontén, T., Aarestrup, F. M., Ussery, D. W., & Lund, O. Benchmarking of Methods for Genomic Taxonomy. *J Cli Microbiol*, **2014**, 52(5), 1529–1539. https://doi.org/10.1128/JCM.02981-13
30. Larsen, M.V., Cosentino, S., Rasmussen, S., Friis, C., Hasman, H., Marvig, R.L., Jelsbak, L., Sicheritz-Pontén, T., Ussery, D.W., Aarestrup, F.M., Lund, O. Multilocus Sequence Typing of Total-Genome-Sequenced Bacteria. *J Cli Microbiol*, **2012,** 50(4), 1355–1361. https://doi.org/10.1128/JCM.06094-11.
31. Florensa, A.F., Kaas, R.S., Clausen, P.T.L.C., Aytan-Aktug, D., Aarestrup, F.M. ResFinder - an open online resource for identification of antimicrobial resistance genes in next-generation sequencing data and prediction of phenotypes from genotypes. *Microb Genom*. **2022,** 8(1):000748. doi: 10.1099/mgen.0.000748. PMID: 35072601; PMCID: PMC8914360.
32. Maquart, M., Le Flèche, P., Foster, G. *et al.* MLVA-16 typing of 295 marine mammal *Brucella* isolates from different animal and geographic origins identifies 7 major groups within *Brucella ceti* and *Brucella pinnipedialis*. *BMC Microbiol* **2009,** 9, 145. https://doi.org/10.1186/1471-2180-9-145.
33. Koren, S., Walenz, B.P., Berlin, K., Miller, J.R., Bergman, N.H., Phillippy, A.M. Canu: scalable and accurate long-read assembly via adaptive k-mer weighting and repeat separation. *Genome Res*. **2017,** 27(5):722-736. doi: 10.1101/gr.215087.116. Epub 2017 Mar 15. PMID: 28298431; PMCID: PMC5411767.
34. Seemann T, A. (n.d.). Abricate. https://github.com/tseemann/abricate.
35. Suárez-Esquivel, M., Baker, K.S., Ruiz-Villalobos, N., Hernández-Mora, G., Barquero-Calvo, E., González-Barrientos, R., Castillo-Zeledón, A., Jiménez-Rojas, C., Chacón-Díaz, C., Cloeckaert, A., *et al*. *Brucella* Genetic Variability in Wildlife Marine Mammals Populations Relates to Host Preference and Ocean Distribution. *Genome Biol. Evol*. **2017,** 9, 1901–1912.
36. Girault, G., Freddi, L., Jay, M., Perrot, L., Dremeau, A., Drapeau, A., Delannoy, S., Fach, P., Ferreira Vicente, A., Mick, V., Ponsart, C., Djokic, V. Combination of *in silico* and molecular techniques for discrimination and virulence characterization of marine *Brucella ceti* and *Brucella pinnipedialis*. *Front Microbiol*. **2024**, 15, 1437408. doi: 10.3389/fmicb.2024.1437408