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Transmission of *Brucella canis* in a canine kennel following introduction of an infected dog

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ABSTRACT

Brucella canis is a zoonotic pathogen and the main causative agent of canine brucellosis. In the Netherlands, *B. canis* had previously only been detected in individual cases of imported dogs. However, an outbreak of *B. canis* occurred for the first time in a cohort of autochthonous dogs in a breeding kennel in 2019. The outbreak began with a positive serological test result of an imported intact male dog showing clinical symptoms of brucellosis. Consequently, urine and blood samples were collected and tested positive for *B. canis* by culture, matrix-assisted laser desorption/ionization – time of flight mass spectrometry (MALDI-TOF MS) and whole-genome-sequencing (WGS). Screening of the contact dogs in the kennel where the index case was kept, revealed that antibodies against *B. canis* could be detected in 23 out of 69 dogs (34 %) by serum agglutination test (SAT). Of the 23 seropositive dogs, *B. canis* could be cultured from the urine and/or heparin samples of 19 dogs (83 %). This outbreak represents the first documented case of transmission of *B. canis* to autochthonous contact dogs in the Netherlands. WGS revealed all *B. canis* isolates belonged to the same cluster, which means the transmission of *B. canis* in the breeding kennel was most likely caused by the introduction of one infected dog. Comparing this cluster with data from other *B. canis* isolates, it also appears that characteristic clusters of *B. canis* are present in several endemic countries. These clusters seem to remain stable over time and may help in locating the origin of new isolates found. This outbreak showed that the international movement of dogs from endemic countries poses a threat to the canine population, while serological screening and WGS proved to be valuable tools for respectively screening and the epidemiological investigation.

1. Introduction

Brucella canis is considered to be an emerging pathogen in Europe and several reports concerning outbreaks in dogs have been published in recent years (Corrente et al., 2010; Dahlbom et al., 2009; Djokic et al., 2023; Gyuranecz et al., 2011). In the Netherlands, *B. canis* was detected for the first time in a dog imported from Romania in 2016 (van Dijk et al., 2021). In contrast to other *Brucella* species, *B. canis* is not a notifiable disease in Europe according to the Animal Health Law. In the Netherlands, national legislation is in place and notifications of suspected cases or positive test results are investigated by the Netherlands Food and Consumer Product Safety Authority. However, follow-up relies on the voluntary cooperation of the dog owner, as there is currently no legal basis for the control of *B. canis*. In addition, the control of *B. canis* is complicated by asymptomatic shedders, diagnostic challenges, unsuccessful antibiotic treatment and the lack of data on the survival in and elimination of the organism from the environment (Cosford, 2018; Santos et al., 2021). Moreover, *B. canis* is a zoonosis and although outnumbered by reports on human infections with other zoonotic *Brucella* species, the close contact between dogs and humans represents another complicating factor to take into account (Djokic et al., 2023).

When notified of a suspected case of B. *canis*, the Dutch authorities will collect samples for confirmatory serology and/or culture of the dog in question and these samples are tested by Wageningen Bioveterinary Research (WBVR), the National Reference Laboratory (NRL) for Brucellosis in the Netherlands. In 2019, the NRL received samples in

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order to perform confirmatory diagnostics for *B. canis* following a positive serological result of a male dog. As outlined in this report, this dog represents the index case of an outbreak of *B. canis* in a Dutch kennel. With regards to the Netherlands, this report describes the first known infection with *B. canis* in a breeding facility with transmission to autochthonous contact dogs.

2. Materials and methods

2.1. Animals and samples

A total of 70 dogs were sampled for serology and a total of 30 dogs for culture over the course of 108 days. Of these 70 dogs, 57 dogs were considered adults and 13 puppies (divided over two litters), and they were Alaskan Malamutes, Karelian Bear Dogs and Laikas. To our knowledge, there was no history of (recent) infertility or abortion in the kennel. For serology, serum samples were collected and for culture, samples included heparin blood and urine samples as well as tissue samples in the case of the four dogs submitted for post-mortem examination. Tissue samples included samples from the spleen, tonsils, lymph nodes (of the head, thorax and abdomen) and depending on the gender of the dog, testicles, prostate, epididymis, ovaria and uterus.

2.2. Serology

Serology was performed by an in-house serum agglutination test (SAT) with the addition of 2-mercapto-ethanol (2-ME) as described by Alton et al. (Alton et al., 1988). An in-house derived positive rabbit anti-*B. canis* control serum validated by the NRL was included as positive control. Titers of <50 were considered to be negative, titers of ≥ 50 and <200 were considered to be inconclusive and titers of ≥ 200 positive.

2.3. Culture and MALDI-TOF MS

For culture, blood samples were collected aseptically and the majority of urine samples were obtained through cystocentesis. All activities concerning the culture of *B. canis* were carried out in a Biosafety Level 3 (BSL-3) laboratory. Urine, blood and tissue samples were cultured using in-house produced serum-dextrose agar (SDA) as well as serum-dextrose broth (SDB), both with added Oxoid™ Brucella Selective Supplement (Thermo Scientific™). A positive control strain of *B. canis* (NCTC 11365) was included in the inoculation of both media at the same day as culturing samples.

Urine samples were centrifuged for 2 min (13.000 g), after which the pellet was resuspended in 500 µl in-house produced cooked meat broth. From the 500 µl cooked meat broth, 1 µl was plated on SDA. The remaining cooked meat broth was added to 45 ml SDB. For the heparin blood samples, 5 ml was added to 45 ml SDB and one drop was plated on SDA. Tissue samples were grinded, homogenized for 1,5 minutes at 6000 RPM (ULTRA-TURRAX® Tube Drive P control), after which 5 ml was added to 45 ml SDB and one drop was plated on SDA.

After inoculation, the SDA and SDB were incubated at 37°C and in 5–10 % CO_2. SDA plates were checked after one week of incubation. One µl of SDB was plated on SDA after one and two weeks of incubation. Suspect colonies were streaked on SDA to ensure pure cultures. Pure cultures of suspect colonies were analyzed by matrix-assisted laser desorption/ionization – time of flight mass spectrometry (MALDI-TOF MS, Bruker Daltonics, Germany) using an in-house database to identify the isolates as *Brucella* species. This in-house database was created to identify *B. canis* and *B. suis,* which were not included in the database of the manufacturer at the time. As this in-house database was not fully validated, isolates identified as *B. canis*, *B. suis* or *Brucella* species were subjected to WGS to confirm their identity at the level of species.

2.4. Whole-genome-sequencing, phylogeny, SNP and mutations

For sequencing, DNA of the isolates identified as *B. canis*, *B. suis* or *Brucella* species by MALDI-TOF MS was purified according to the protocol for DNA Purification from Gram-Negative Bacteria Using the Gentra Puregene Yeast/Bact. Kit (Gentra® Puregene® Handbook, 2011). Illumina's DNA Prep protocol following the manufacturer's protocol was used for constructing deep sequencing libraries. Deep sequencing libraries were analyzed using Illumina's MiSeq PE150 running kit. Raw sequence reads were trimmed for artefacts using the BBTools suite (Brian Bushnell (2014)). BBMap (sourceforge.net/projects/bbmap/) and the draft genomes were constructed using the Shovill pipeline with the assembler SKESA version 1.0.9 (Souvorov et al., 2018). One isolate per dog (n=24) was included in the phylogenic tree. In addition, sequence data from *B. canis* isolates (n=16) that were obtained by the Dutch National Reference Laboratory for Brucellosis prior to the outbreak were added to the phylogenic tree. All assemblies (n=40) were uploaded to the European Nucleotide Archive under study-ID PRJEB76235 with sample-IDs ERS20174068–107. A maximum likelihood tree was build using the Nextstrain pipeline (version nextstrain.cli 2.0.0.post1). To prepare the data, a merged-vcf-file was made that contained the variants per strain against the reference (GCF_000018525.1). Mapping was done by bwa (version 0.7.17-r1188), pilon (version 1.23) was used to create separate vcf-files and they were merged with vcf-merge. The phylogeny was built with IQ-TREE (version 2.0.3) on a file containing the informative sites filtered from the merged vcf-file, using the GTR model and the following specifications (-ninit 2 -n 2 -me 0.05 -nt 1). To construct the phylogeny, Nextstrain created files with all SNPs (single-nucleotide polymorphisms) and mutations (changes at the amino acid level) identified in the samples compared to the reference genome of *B. canis* ATCC23365 (GenBank accession number GCA_000018525.1). SNPs present at the base of each cluster were collected from the Nextstrain phylogeny and this set was spatially visualized with Proksee (Grant et al., 2023). In addition, a heatmap of all filtered mutations was created in R with pheatmap (Raivo Kolde (2019). pheatmap: Pretty Heatmaps. R package version 1.0.12. https://CRAN.R-project.org/package=pheatmap), in which rows were divided into four blocks based on similarity of mutations.

3. Results

3.1. Case description

Index case In January 2019, the Dutch authorities were notified by a veterinary laboratory of a positive serological test result concerning *B. canis* (titer: 200). The sample in question was collected from an intact male Laika dog showing clinical symptoms including back pain, loss of appetite and lameness. The dog had been tested positive for anaplasmosis previously, but an insufficient response to treatment led to further testing and the dog was subsequently diagnosed with canine brucellosis. The dog had been imported from Russia two years earlier by a breeder in the Netherlands. Following the notification, urine and blood samples of the seropositive dog were collected by the Dutch authorities and sent to the National Reference Laboratory for confirmation (Day 0, refer to Table 1). Because the dog was fed raw meat, a SAT for *Brucella suis* was also performed, in addition to a SAT for *B. canis* and bacteriological culture. Following confirmation of the positive serological test result for *B. canis*, the Dutch authorities decided not to wait for the results of the culture, but initiated an epidemiological investigation, starting with the serological screening of contact dogs.

3.1.1. Epidemiological investigation

Seven days after the sampling of the index case (Day 7, refer to Table 1) urine and heparin blood samples were collected from three female dogs (BITCH01, BITCH02 and BITCH03), as it was said that they had been imported together with the index case. The animals appeared

Table 1
Overview of the samples collected during the outbreak of *B. canis* described starting at the day of the collection of the official samples of the index case (Day 0). Dogs were tested serologically (by serum agglutination test) or by culture (heparin blood, urine or tissue samples) or both (depending on the result). Dogs were found to be positive by culture based on a positive urine sample only (Column 4), a positive heparin blood sample only (Column 5) or based on both urine and heparin blood samples testing positive (Column 6). NT: not tested (either not at all or at that particular time during the outbreak). *The positive tissue samples (n=11) originated from the four dogs, of which tissue samples (n=31) were collected.

Day of Sampling	DOG ID	Positive samples/ tested samples by SAT	Positive urine samples/ tested samples by culture	Positive heparin blood samples/ tested samples by culture	Positive urine and heparin blood samples/ tested samples by culture	Positive tissue samples/ tested tissue samples by culture
Day 0	INDEX CASE	1/1	1/1	0/1	0/1	NT
Day 7	BITCH01 BITCH02 BITCH03	3/3	0/3	2/3	0/3	NT
Day 25	DOG01 – DOG53	20/53	NT	NT	NT	NT
Day 33	PUPPY01 PUPPY02 PUPPY03 PUPPY04 PUPPY05 PUPPY06 PUPPY07 PUPPY08 PUPPY09 PUPPY10 PUPPY11 PUPPY12 PUPPY13	0/13	NT	NT	NT	NT
Day 33	DOG01 DOG02 DOG03 DOG05 DOG06 DOG07 DOG08 DOG09 DOG10 DOG17 DOG18 DOG19 DOG20 DOG22 DOG24 DOG27 DOG35 DOG36 DOG37 DOG38 DOG40 DOG42	NT	1/22	8/22	8/22	NT
Day 108	DOG15 DOG23 DOG33 DOG44	NT	0/4	0/4	1/4	11/31*
Total positive samples/samples tested		24/70	2/26	10/26	8/26	11/31
Positive dogs/dogs tested		24/70	2/26	10/26	8/26	4/4

to be healthy and in good condition. Further investigation and visits to the breeding facility revealed that the three bitches had been imported individually and not together with the index case. However, one of the three bitches appeared to have the same sire as the index case. Of the 72 dogs kept by the breeder, two of them showed signs of impaired health seemingly unrelated to brucellosis (cachexia and blindness, respectively). The owner decided to euthanize these two dogs, together with the index case and the three bitches. Samples were not collected from the two dogs showing cachexia and blindness. Blood samples of the remaining adult dogs (n=53) at the kennel were collected for serological screening (Day 25, refer to Table 1). After contacting the breeder in Russia, the authorities learned that the sire of the index case (and of one the female dogs) had been tested positive for *Brucella canis*. Unfortunately, further information including the diagnostic method used was unavailable.

A second round of screening (on Day 33, refer to Table 1) included the litters (a total of 13 puppies) present at the dog kennel. At the same time of sampling the puppies (on Day 33, refer to Table 1), urine and heparin blood samples were collected for culture from the 20 dogs that tested seropositive in the first round of screening (which took place on Day 7, refer to Table 1) and from two seronegative dogs (DOG19 and DOG27), that appeared to be in bad condition.

At first, the owner decided to only euthanize the seropositive dogs. When it became clear that the other dogs, although seronegative, would pose a risk for making a new start with the kennel, the owner decided to euthanize all remaining dogs. The bodies of four randomly selected

adult dogs, which tested seronegative on Day 25, were transferred to the National Reference Laboratory for post-mortem examination and samples of several tissues were collected for culture.

The authorities also made efforts to trace any dog that was purchased from the breeder since the first dog was imported from Russia and introduced to the breeding facility in 2015. Due to an incomplete administration on the side of the breeder, only 38 owners were contacted, while an estimated 147 dogs had been sold in the period of interest. Owners were urged by an information letter to have their dog tested for *B. canis* antibodies, but to our knowledge, none of the owners complied.

3.2. Serology

The serum sample of the index case was found negative for antibodies against *B. suis*, but positive for antibodies against *B. canis* (titer: ≥400). BITCH01, BITCH02 and BITCH03 also tested positive for antibodies against *B. canis*, with titers ranging from 50 to >=400. Of the 53 adult dogs (DOG01 – DOG53) that were included in the serological screening, 20 tested positive for *B. canis* antibodies. Sixteen of these dogs showed a titer of >=400, three of them a titer of 200 and one a titer of 50. The thirteen puppies (PUPPY01 – PUPPY13) that were sampled, tested negative for antibodies against *B. canis*. These two litters shared the same sire (DOG23), but different dams, DOG22 and DOG46 respectively. DOG22 was amongst the sixteen dogs which tested positive for antibodies against *B. canis*.

3.3. Culture and MALDI-TOF MS

MALDI-TOF MS was able to identify 23 isolates as *B. canis* and one isolate as *Brucella* spp. following the culture of a total of 88 samples belonging to 30 dogs. The urine sample of the index case was found positive for *Brucella* species, while no *Brucella* bacteria were detected in the heparin blood sample. In addition, the heparin blood samples of two of the three seropositive bitches (BITCH01 and BITCH02) were tested positive for *B. canis*, while culturing the urine samples yielded negative results. Of the 20 seropositive dogs sampled on Day 33, 17 were positive for *B. canis*, including DOG22, the dam of one of the two litters. The two seronegative dogs that were sampled together with the 20 seropositive dogs, tested negative, despite being in a bad condition. Of the four dogs subjected to post-mortem examination on Day 108, a total of 31 tissue samples were cultured, of which 11 samples belonging to four different dogs were found to be positive for *B. canis*. Only one of these four dogs was found positive for *B. canis* based on the culture of urine and heparin blood samples: *B. canis* could not be cultured from the urine and heparin blood samples of the other three dogs.

3.4. Whole-genome-sequencing, phylogeny, SNPs and mutations

The results of the MALDI-TOF MS were confirmed by WGS as all isolates identified by MALDI-TOF MS as *B. canis* or *Brucella* spp. were identified as *B. canis* by WGS. Analysis of the maximum-likelihood phylogeny revealed that all *Brucella* isolates detected during the outbreak belonged to one cluster, which suggests that there was a single introduction event or multiple introductions from a common source (Fig. 1). Additionally, based on the sequence data of the set of isolates obtained from previous submissions by the NRL prior to the outbreak, isolates seem to cluster per country (Fig. 1). This relationship seems to be stable, as isolates detected in different years within the same country (for example, the isolates from dogs from Romania) still show high

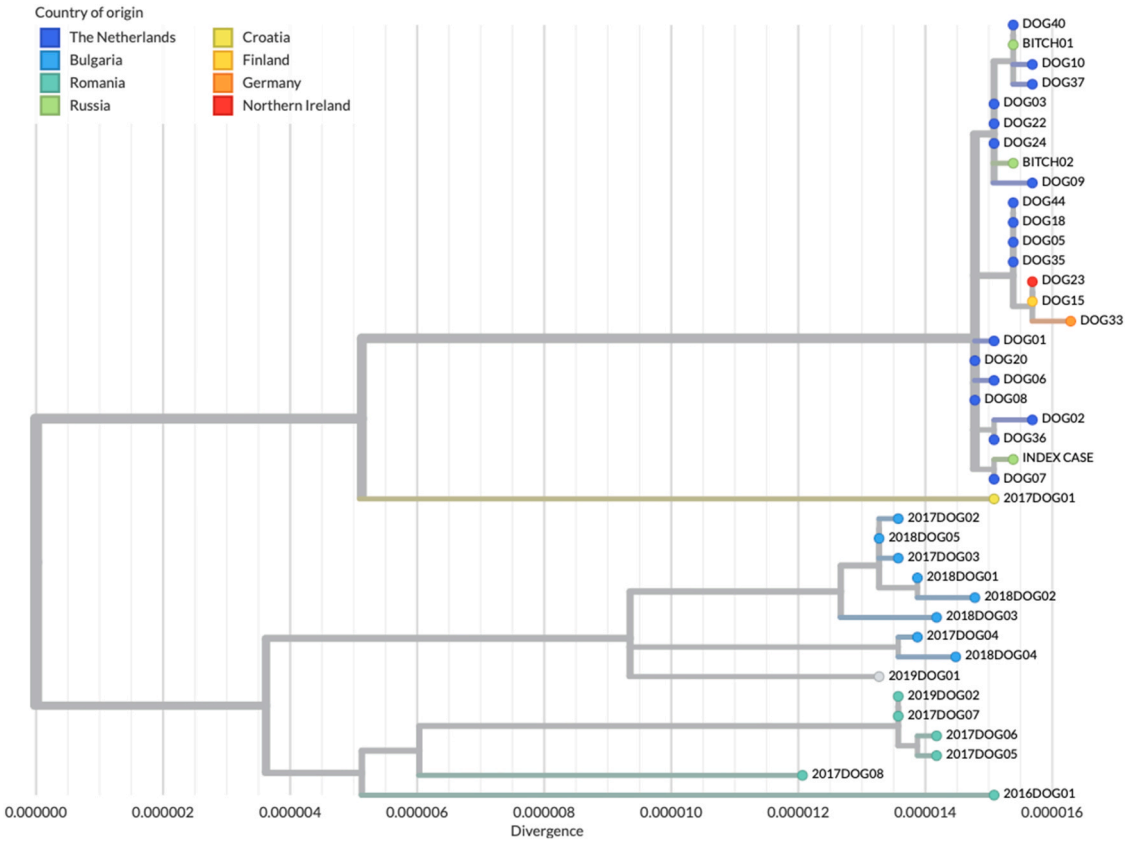

Fig. 1. : Maximum-likelihood phylogeny of whole genome *Brucella canis* sequences made with IQ-TREE within Nextstrain, using the GTR model. Branches and tips are colored by country of origin (The Netherlands, Bulgaria, Romania, Russia, Croatia, Finland, Germany and Northern Ireland). Isolates cultured from previous submissions by the Dutch NRL for Brucellosis prior to the outbreak have been named after the year of submission. Outbreak isolates do not contain the year in their label. Isolate 2016DOG01 represents the index case of the case report from 2016 by van Dijk et al. (van Dijk et al., 2021).

similarity in sequence data.

To examine the spatial distribution of SNPs present at the base of each cluster over the genome, SNPs were compared between the reference *Brucella canis* ATCC 23365 strain and the sequences of isolates with Dutch, Bulgarian, Croatian and Romanian origin (Fig. 2). In total, 89 SNPs were present in all dogs of each cluster, leading to 43 changes at amino acid level. In general, these SNPs are evenly distributed over the genome indicating there are no mutational hotspots linked to the separation over the different geographical locations, or to mobile genetic elements or prophage regions in the genome.

In order to look at a more functional level, we depicted all amino acid mutations within a heatmap (Fig. 3). The blocks defined based on similarity, group similar to the clusters within the phylogeny, thus creating an overview of which mutations belong to which cluster. Although some mutations are located in regulatory genes (e.g., *lexA*), it is yet unclear which functional changes can be attributed to these mutations. Based on this information, no clear difference in function between the groups can be determined at this moment. In general, the total mutational rate at amino acid level appeared to be 1.5 times higher in chromosome II (74 mutations per Mb) compared to chromosome I (48 mutations per Mb).

4. Discussion

Transboundary spread of *B. canis* and more specific the introduction of *B. canis* in the Netherlands through the import of infected dogs from *B. canis*-endemic areas, has been described quite recently (van Dijk et al., 2021). While the risk of the import of dogs from areas where *B. canis* is considered endemic has been acknowledged, transmission of *B. canis* from an index case (with a history of travel from an endemic area) to contact dogs had not been reported in the Netherlands. This paper is the first publication describing an outbreak of *B. canis* in autochthonous dogs as well as in a dog breeding facility in the Netherlands. Since the outbreak described involves a presumed index case, based on the presence of one cluster and the alleged *B. canis* infection of the sire of this imported dog, this is also the first publication on assumed transmission of *B. canis* from an imported index case to contact dogs in the Netherlands. However, the introduction of *B. canis* via other routes or animals (for example, through the half sibling of the index case that was imported six months after the import of the index case) cannot be excluded.

Serological testing (by SAT) proved to be an adequate tool for screening. Of the 24 seropositive dogs, *B. canis* was cultured from the urine and/or heparin blood sample of 20 dogs (Table 2). Interestingly, the four dogs (DOG15, DOG23, DOG33 and DOG44) that were subjected to autopsy on Day 108 and were tested positive for *B. canis* by culture, had been tested negative for antibodies on Day 25. This means that either the dogs became infected with *B. canis* after the first round of sampling or that these dogs had not produced detectable antibodies against *B. canis* after infection at the time of sampling. Seroconversion may take up to 3 months and this is also one of the reasons why a second sample is needed to confirm the presence or absence of antibodies (Cosford, 2018; Djokic et al., 2023). The majority of the seronegative dogs were not tested again after the first round of sampling. In hindsight, it would have been interesting in the light of these seronegative, culture positive dogs, to repeatedly check for either *B. canis* or antibodies against *B. canis* in dogs initially tested negative to get more insight in the incubation time of *B. canis* and/or the time to seroconversion. This also holds true for the seronegative puppies of DOG22, which tested positive for *B. canis* both by serology as well as by culture.

The challenges regarding the diagnostics of *B. canis* are illustrated by the four dogs discussed earlier (DOG15, DOG23, DOG33 and DOG44), of which tissue samples as well as heparin blood and urine samples tested positive for *B. canis* by culture. Although *B. canis* was detected in at least one tissue sample in all four dogs, urine and heparin blood samples were negative for three of the four dogs. As urine and heparin blood samples are the samples of choice for culturing, these three dogs (DOG23, DOG33 and DOG44) would have been considered negative for *B. canis* according to routine procedures for culture. Also, regarding these three dogs, *B. canis* was detected only in a spleen sample, a sample from a thoracal lymph node and a tonsil sample, respectively. The bacterial load in samples other than urine and genital discharges has been described to be low (Djokic et al., 2023). In addition, the distribution in tissues may vary (de Souza et al., 2018). (False-)negative culture results of both urine and heparin blood samples as well as the apparent need to collect (multiple) tissue samples of infected animals complicate the epidemiological investigation during an outbreak.

Despite the fact that multiple dogs tested positive for *B. canis* (either by culture or by serology), precautionary measures to protect the other

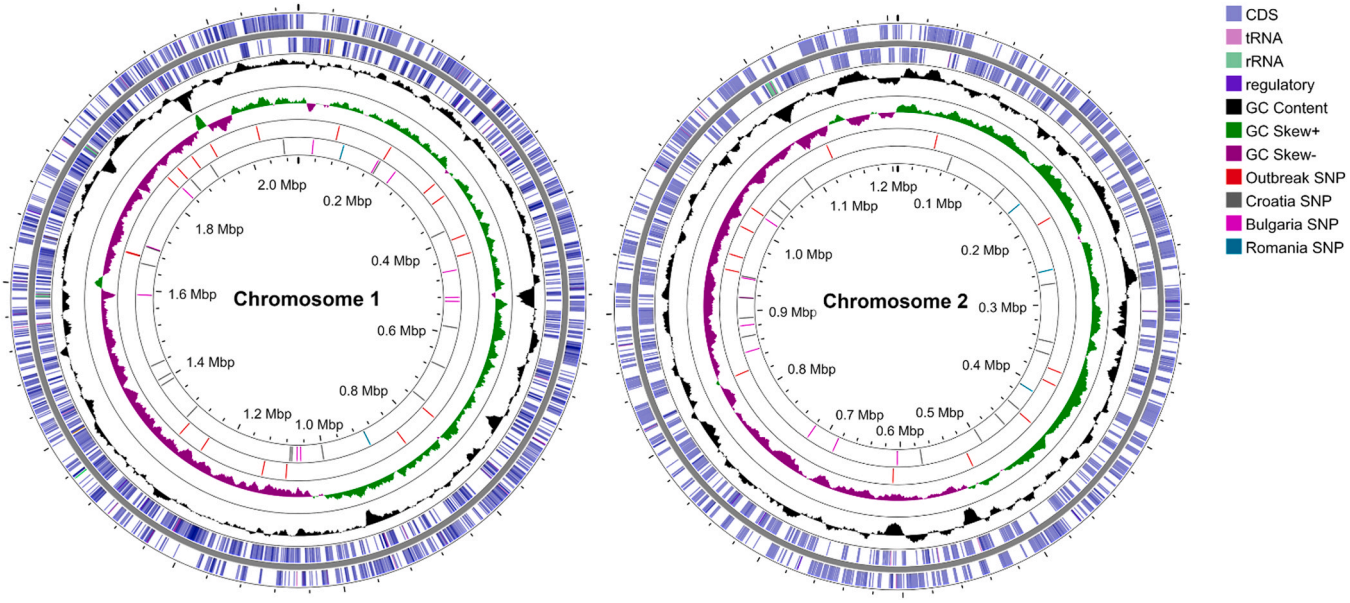

Fig. 2. Circular representation of the two chromosomes of *Brucella canis*. Circles from outside to inside represent the position of protein-coding genes, and different RNAs (tRNA, rRNA and regulatory RNA) on the positive and negative strand. Ring 3 and 4 show the GC content and GC skew. Ring 5 shows the positions of the SNPs present in the Dutch outbreak isolates, while ring 6 shows the SNPs in the strains from Bulgaria (pink), Croatia (grey) and Romania (green blue).

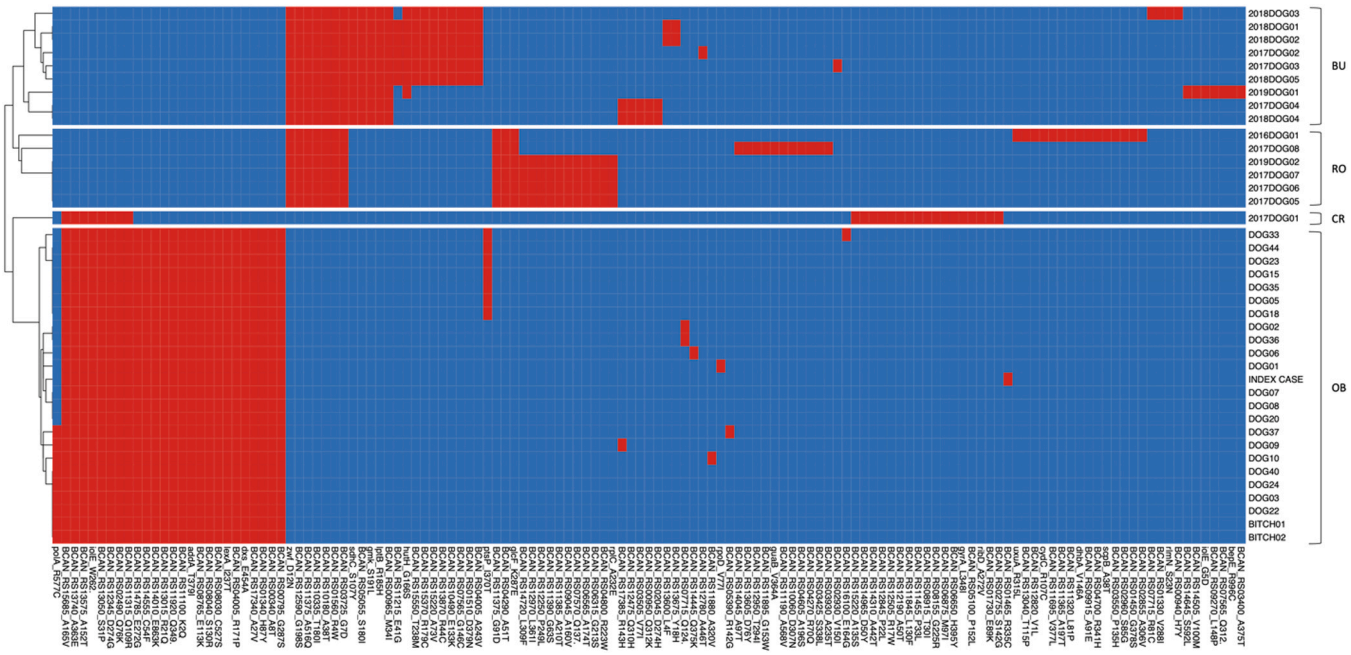

Fig. 3. : Heatmap of the mutations per *Brucella canis* isolate. A red square indicates the mutation is present and a blue square that it is absent. Rows were divided into four blocks based on similarity; they are identical to the clusters found within the phylogeny. Abbreviated labels of the blocks stand for: OB = outbreak, BU = Bulgaria, RO = Romania and CR = Croatia.

Table 2
Comparison of results yielded by testing the dogs by serology and by culture. Note that the time of sampling for serology or culture may differ.

	Dogs tested positive by culture	Dogs tested negative by culture
Dogs tested positive by SAT	20	4
Dogs tested negative by SAT	4	2

dogs residing in the facility were or could not be taken. All dogs, although living in groups of two to four dogs, had access to the same outdoor area, which was covered with sand. Feces was removed from the outdoor area, but not on a regular basis. As pointed out in a recently published review (Djokic et al., 2023), it is unclear under which environmental conditions *B. canis* may be able to survive and for how long. For other *Brucella* species, survival up to months has been described under both natural as well as experimental conditions (Aune, 2011; Scholz et al., 2008). In this case, we cannot say whether and to what extent indirect transmission of *B. canis* played a role during the outbreak.

As illustrated by this paper, WGS can provide insight into the source of the outbreak. Not only does the presence of one cluster point in the direction of one introduction (or multiple introductions from the same source), which is valuable information when conducting an epidemiological investigation. There also appear to be characteristic clusters of *B. canis* in several countries. These clusters seem to remain stable over time and may help in locating the origin of the strain of *B. canis*. The SNPs underlying these differences between clusters seem equally distributed over the genome and no clear function can be linked to these mutations, which is consistent with neutral mutations that occur between subpopulations of similar and near geographical locations. Moreover, our data reveals a higher mutational rate in chromosome 2 compared to chromosome 1. This supports the hypothesis that chromosome 2 is more dynamic than chromosome 1 due to a higher prevalence of essential genes on chromosome 1 (Wattam et al., 2009). Unfortunately, no sequence data of Russian *B. canis* isolates were available at the time of analysis, which would have allowed to compare the sequences with an assumed Russian origin with validated data. However, given that *B. canis* is not endemic in the Netherlands and that it is unlikely that the dogs originating from Germany, Finland and Northern-Ireland were responsible for the introduction (non-endemic countries, serologically negative at Day 25, no clinical signs, no link with *B. canis* infected dogs), introduction through the dogs imported from Russia (the index case and/or its (half-)sibling) is most probable in this case.

The outbreak described was most likely caused by a single introduction of *B. canis,* which can be attributed to the index case and involved *B. canis* infections in 28 out of 69 contact dogs based on the results of serology and/or culture. This case is illustrative for the risk of introducing *B. canis* through the import of dogs from endemic areas and underlines the need for screening before import. It is estimated that 1000 dogs (either illegally or legally) are imported into the Netherlands on a weekly basis, of which an unknown number originate from countries where *B. canis* is endemic. It is not known to which extent these imported dogs are acting as a source of transmission of *B. canis* for other dogs in the Netherlands, as cases are most likely underreported due to lack of screening, unawareness among dog owners and veterinarians and the subclinical nature of some *B. canis* infections.

At the time of the outbreak, no human cases of *B. canis* had been reported, but the zoonotic aspect of this bacterium and the (occupational) risk for the owner of the dogs as well the staff of the veterinary practice involved was taken into account. On a voluntary basis, one of the pregnant staff members was tested negative for antibodies against *B. canis* by the NRL (SAT, not validated for humans). However, the zoonotic risk associated with importing dogs from endemic areas was illustrated by the first human case of *B. canis* only two years later (Kolwijck et al., 2022). The screening of dogs from endemic areas as well as the awareness amongst dog breeders is highly recommended to prevent outbreaks of *B. canis*. However, to do so, uniform protocols for diagnosing *B. canis*, both in humans and animals are needed, based on experimental studies as well as the reports of outbreaks, like the one described here.

Ethics statement

All animals in this study were sampled as part of the outbreak investigation as commissioned by the Netherlands Food and Consumer Product Safety Authority as required by and according to the European Animal Health Law and Dutch national regulations on control of brucellosis.

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CRediT authorship contribution statement

Ad Koets: Writing – review & editing, Supervision, Resources, Project administration, Investigation, Data curation, Conceptualization. **Heather Graham:** Writing – original draft, Investigation, Data curation, Conceptualization. **Marleen van der Most:** Writing – review & editing, Investigation, Data curation, Conceptualization. **Reina van der Heide:** Writing – review & editing, Investigation. **Lucien van Keulen:** Writing – review & editing, Investigation. **Annemieke Dinkla:** Writing – review & editing, Investigation. **Frank Harders:** Writing – review & editing, Investigation. **Andries A. Kampfraath:** Writing – review & editing, Visualization, Investigation, Formal analysis. **Vanessa Visser:** Writing – review & editing, Investigation, Data curation. **Robin Ruuls:** Writing – review & editing, Investigation. **Alieda van Essen-Zandbergen:** Writing – review & editing, Investigation. **Marielle H. van den Esker:** Writing – review & editing, Visualization, Investigation, Formal analysis.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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