First Isolation of Brucella canis *from a breeding kennel in Italy*

Fabrizio De Massis[1], Flavio Sacchini[1], Daniela Averaimo[1*], Giuliano Garofolo[1], Pierdavide Lecchini[2], Luigi Ruocco[2], Roberto Lomolino[2], Ugo Santucci[2], Elisa Sgariglia[3], Silvia Crotti[3], Antonio Petrini[1], Giacomo Migliorati[1], Nicola D'Alterio[1], Stefano Gavaudan[3] and Manuela Tittarelli[1]

[1]National and OIE Reference Laboratory for Brucellosis, Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise "G. Caporale", Teramo, Italy.
[2]Direzione generale della sanità animale e dei farmaci veterinari, Ministero della Salute, Roma, Italy.
[3]Istituto Zooprofilattico Sperimentale dell'Umbria e delle Marche "Togo Rosati", Perugia, Italy.

[*]Corresponding author at: National and OIE Reference Laboratory for Brucellosis, Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise "G. Caporale", Campo Boario, 64100 Teramo, Italy.
E-mail: d.averaimo@izs.it.

Veterinaria Italiana 2021, **57** (3), 215-226. doi: 10.12834/VetIt.2497.15848.1
Accepted: 01.09.2021 | Available on line: 31.12.2021

Keywords
Brucella canis,
Isolation,
Dogs,
Serology,
cgMLST.

Summary
Brucella canis has been isolated for the first time in Italy in a commercial breeding kennel. It was diagnosed after a deep investigation related to the onset of reproductive disorders. Animals were tested with direct and indirect techniques. The agent was first detected in two Chihuahua aborted foetuses by direct culture. Further, it was also isolated from blood samples of dogs hosted in the kennel, which also showed reaction to conventional serological tests (microplate serum agglutination test). The isolates were identified as *B. canis* by standard microbiological methods and a Bruce-ladder multiplex PCR. To investigate the genomic diversity, whole genome sequencing was used, applying the core genome Multilocus Sequence Typing (cgMLST). In a first round of serological testing performed on 598 animals, 269 (46.1%) tested positive. In the second round of laboratory testing carried out 4-5 weeks apart, the number of serologically positive dogs was 241 out of 683 tested (35.3%), while the number of dogs positive to isolation was 68 out of 683 tested (10.0%). The PCR showed a lack of sensitivity when compared to direct isolation. The epidemiological investigation did not identify the source of the infection, given the time elapsed from the onset of abortions to the definitive diagnosis of *B. canis* infection in the kennel. The genomic analyses featured the strains as ST21 and, according to the cgMLST, revealed the presence of a tight cluster with a maximum diversity of four allelic differences. The observed limited genomic variation, largely within the known outbreak cut-offs, suggests that the outbreak herein described was likely caused by a single introduction. Moreover, in a broader scale comparison using the public available genomes, we found that the closest genome, isolated in China, differed by more than 50 alleles making not possible to find out the likely origin of the outbreak. The lack of updated data on *B. canis* genome sequences in the public databases, together with the limited information retrieved from the epidemiological investigations on the outbreak, hampered identification of the source of *B. canis* infection.

Introduction

Brucellosis is a zoonosis, widespread all over the world, caused by bacteria belonging to the genus *Brucella*. The most clinically relevant *Brucella* species are *Brucella melitensis, B. abortus, B. suis* and *B. canis*. They tend to be host-adapted, although infections may occur in other animal species, including humans (Michaux-Charachon *et al.* 2002).

First identified in 1966 (Carmichael 1966), *B. canis* is a Gram-negative non-motile aerobic intracellular coccobacillus with rough colony morphology when grown on artificial medium. Dogs and wild *Canidae* are the only animal species that act as reservoirs of *B. canis* under natural conditions (Shin and Carmichael 1999).

Canine Brucellosis due to *B. canis* is a contagious disease characterized by abortions in females and

epididymitis, testicular atrophy, prostatitis and infertility in males (Wanke 2004). The disease is insidious, and many dogs do not have prominent signs, otherwise some dogs with generalized infection could show uveitis, discospondylitis and other more chronic conditions such as lymphadenomegaly, osteomyelitis, polyarthritis, meningoencephalitis, and pyogranulomatous dermatitis (Carmichael and Greene 2013). The disease is of particular importance for dog breeders since infection with *B. canis* usually ends a dog's reproductive career (Carmichael 1990).

The most common routes of transmission of *B. canis* to humans are through contact with infected dogs, which may disseminate the bacteria with their secretions for many months after bacteraemia has ceased, and through direct laboratory exposure (Carmichael and Shin 1996).

Dog to dog transmission occurs during breeding, or through oronasal contact with reproductive discharges following abortions. *B. canis* may also be shed with urine, feces, and nasal and ocular secretions. Pups may be infected in utero or perinatally (Carmichael and Greene 2013).

These bacteria may be transmitted either by the venereal or oral route, more frequently infection occurs following contact with abortive material. In males, urine and seminal fluid represent an important source of infection (George *et al.* 1979). Prolonged bacteraemia is a typical sign of canine brucellosis that persists from 6 months to 5 years (Carmichael *et al.* 1984).

Prevalence and spreading of *B. canis* infection in canine populations depend upon several individual factors, including age and reproductive status (Carmichael and Greene 2013, Kaufmann and Petersen 2019). In endemic countries, infection rates are typically higher in stray animals, as they are more likely to be reproductively intact and active compared to owned pets. Breeding kennels can also be sites of higher-than-normal infection rates (Carmichael and Greene 2013).

Identifying brucellosis-infected dogs is often challenging. Definitive diagnosis requires the culture of *B. canis* from the blood of infected dogs, a process that has a relatively low sensitivity, is time-consuming, and somewhat technically impractical to apply to large populations of dogs. Some serological methods have been developed as commercial kits and test services are available at diagnostic laboratories. False-positive and false-negative results may occur with these tests (Keid *et al.* 2009), however, in many situations they are the tests of choice for screening larger populations, such as stray dogs or those entering shelters (Carmichael and Greene 2013).

Unlike many bacterial infections, canine brucellosis is not amenable to treatment with antibiotics (NASPHV 2012, Carmichael and Greene 2013). Its intracellular persistence makes *B. canis* a difficult target for antibiotics, despite its susceptibility *in vitro*. Treatment failures or relapses are common (Carmichael and Greene 2013), leading some entities to recommend that infected dogs, regardless of whether they are treated or not, should not be rehomed from infected facilities (USDA 2015).

Canine brucellosis due to *B. canis* is considered a zoonosis (Blankenship and Sanford 1975). The disease is underestimated in man due to general lack of serological testing facilities and misconceptions concerning its prevalence. Confirmed cases of human illness due to *B. canis* are relatively uncommon, with roughly 50 cases identified in the US since 1973 (Daly *et al.* 2020). However, because of the vague symptoms and effects in infected people, it is likely that human cases of canine brucellosis are under-diagnosed and underreported (NASPHV 2012). Those working closely with potentially infected animals, such as in breeding kennels or with stray animals, are considered at higher risk than others (Hensel *et al.* 2018). Culture-positive cases have been reported in laboratory personnel, animal technicians and persons known to have close and frequent contact with infected dogs (Carmichael *et al.* 1980).

B. canis is considered endemic in Southern USA, in Central and South America and in Mexico (Flores-Castro *et al.* 1977, Brower *et al.* 2007, Küster de Paula Dreer *et al.* 2013, Krueger *et al.* 2014, Keid *et al.* 2017). It has been also reported in Canada (Cosford *et al.* 2018). Infections with *B. canis* have been reported from Asian countries such as China (Di *et al.* 2014), Japan (Hayashi and Ysayama 1977), India (Yoak *et al.* 2014) and from African countries such as Nigeria (Cadmus *et al.* 2011) and Zimbabwe (Chinyoka *et al.* 2014). New Zealand and Australia appear to be free of *B. canis*; however, Australia has reported some *B. suis* infections in dogs, mainly in animals used to hunt feral pigs. (Mor *et al.* 2016, Rovid Spickler 2018, Gardner and Reichel 1997). Cases have been reported also from European countries, such as Austria (Hofer *et al.* 2012), Germany (Von Kruedener 1976, Nöckler *et al.* 2003), Hungary (Gyuranecz *et al.* 2011), Sweden (Holst *et al.* 2012, Kaden *et al.* 2014), Switzerland (Egloff *et al.* 2018), and the United Kingdom (Taylor 1980, Whatmore *et al.* 2017). The only report recorded in Italy so far has been a presumptive *B. canis* infection in a dog with chronic prostatitis and discospondylitis, detected by PCR (Corrente *et al.* 2010).

Aims of this paper are to report the first isolation of *B. canis* from a commercial breeding kennel in Italy, to describe the case, and to inform about results of testing.

Materials and methods

Background and diagnostic screening

In April 2020, two Chihuahua aborted foetuses were submitted to the Ancona Laboratory of the Istituto Zooprofilattico Sperimentale dell'Umbria e delle Marche (IZSUM) for the detection of canine reproductive pathogens. These specimens were collected from a kennel where several months of abortion, infertility and reproductive disorders were reported by the private veterinarians caring for the kennel. At time of sampling, the kennel hosted approximately 600 dogs, mostly Chihuahua breed, but also some Pomeranian, Maltese and Toy Poodle breeds. Samples collected from the two foetuses were subjected to standard bacteriological investigations and molecular tests for the following pathogens: Canine Herpes virus (CHV; real-time PCR); *Brucella* spp. (real-time PCR); *Leptospira* spp. (real-time PCR); *Chlamydia* spp. (real-time PCR); *Mycoplasma* spp. (PCR); Canine Parvovirus (CPV; real-time PCR). *Brucella* spp. were isolated in sheep blood agar, after incubation overnight at 37 °C (± 2 °C). Cultures were carried out from brain, spleen, stomach and lung. Identification of *Brucella* genus was confirmed by MALDI-TOF and PCR, both from colonies and pooled viscera. The strain isolated was submitted for identification to the National Reference Centre for Brucellosis (NRC), Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise (IZSAM), where Bruce-ladder assay identified *B. canis* (OIE 2016). Following the confirmation of *B. canis* isolation in the kennel, a bacteriological and serological survey was carried out, to investigate on the magnitude of the outbreak. Samples were collected by Local Veterinary Services according to Italian and European regulations for animal welfare. Samples were taken from the radial vessel with the Vacutainer™ system in anticoagulant tubes, preventing blood from clotting. After collection, blood samples were kept refrigerated until delivered to the laboratory and then stored at 4 ± 2 °C until analysis.

Population data

All dogs were identified with a microchip; data regarding age, gender, and breed were collected from the National and Regional Databases for the identification and registration of dogs.

Serological investigation

After the confirmation of *B. canis* outbreak in the kennel, a first round of serological sampling was carried out on 598 animals and submitted to the NRC, IZSAM. Four-five weeks later, a second round of sampling involved 683 animals and this time both serum and EDTA blood were collected from each animal. Overall, serum samples were collected from 683 dogs and submitted to the NRC, IZSAM. Serological analyses were carried out using a microplate serum agglutination test (mSAT). The test was carried out modifying the tube agglutination test described by Alton and colleagues (Alton *et al.* 1988), and volumes were adapted to be performed in 96-well U-shaped microplates. Briefly, *B. canis* strain RM66 was used to prepare mSAT antigen as previously described (Alton *et al.* 1988). Before testing, serum samples were diluted 1:10 in Tris-maleate buffer (TMB) pH 9.0 ± 0.5. The assay was performed by dispensing equal volumes (50 µl) of sera 2-fold serially diluted with TMB and *B. canis* antigen in a 96 well U-shaped microplate, to obtain a final dilution ranging from 1:20 to 1:640. Plates were incubated at 37 ± 2 °C for 48 h. Samples displaying 100% agglutination at a dilution ≥ 1:20 were considered as positive. Serology titres were indicated as the highest dilution of serum showing 100% agglutination.

Furthermore, in order to acquire information on specificity of mSAT, a panel of 143 samples from owned dogs non-related to the outbreak was also tested with this method.

Bacteriological investigation

A total of 683 dogs of different breed were sampled and a whole blood sample was taken from each of them and submitted to the NRC, IZSAM. Whole blood samples were cultured for detection of *Brucella* spp. according to the literature methods (Carmichael and Greene 2006, CFSPH 2018, GDA 2020). Briefly, samples were both streaked onto selective Farrell's *Brucella* Agar (IZSAM) and inoculated in enrichment broth supplemented with equine serum (*Brucella* Broth, IZSAM). All media were incubated at 37 ± 1 °C. Subcultures in Farrell's *Brucella* Agar of all the broth cultured samples were made weekly for a month. Plates were examined daily for evidence of growth and were considered negative when no colonies were seen neither from direct culture nor from all subcultures. Typical *Brucella* spp. colonies were subjected to specific PCR assay for species identification (OIE 2016).

PCR assay

DNA was extracted from all the samples (whole blood and suspected colonies) using the Maxwell® 16 Blood & Cell DNA Purification Kit (Promega Italia Srl, Milan, Italy), following the manufacturer's specifications. After extraction, DNA was collected in DNA/RNA free tubes and stored at 4 ± 2 °C until

analysis. The reaction mix was prepared using the *Brucella* genus (all species) Genesig™ Advanced Kit (Genesig, York House, School Lane, Chandler's Ford, UK) in a final volume of 20 µl consisting of 5 µl of extracted DNA and 15 µl of master mix, according to the manufacturer's instructions. The samples were analyzed by real-time PCR. PCR was run in a QuantStudio™ 7 Pro real-time PCR System (Applied Biosystems, CA, USA) under the following conditions: enzyme activation step at 95 °C for 2 min, 50 cycles of denaturation at 95 °C for 10 s and data collection at 60 °C for 60 s. A positive control (K+) and a No-Template Control (NTC) were included in each run. Data were analyzed by Design and Analysis Software 2.4.3.

Whole-genome sequencing (WGS)

A total of 67 *B. canis* strains each isolated from a single dog were submitted to WGS. The genomic DNA extracted from bacterial colonies was quantified with the Qubit fluorometer (QubitTM DNA HS assay; Life Technologies, Thermo Fisher Scientific Inc., Waltham, MA, USA). Sequencing libraries were prepared using Nextera XT library preparation kit (Illumina Inc., San Diego, CA, USA) according to the manufacturer's instructions. The libraries were sequenced using the Illumina NextSeq 500 platform, producing 150-bp paired-end reads. After demultiplexing and removal of adapters, reads were trimmed from 50 and 30 ends using Trimmomatic tool version 0.36 to discard the nucleotides with quality scores of less than 25. Reads shorter than 36 bp were automatically discarded. Scaffolds were assembled with SPAdes version 3.11.1 with the careful option selected (Bankevich *et al.* 2012). Read sequences were submitted to Sequence Read Archive of the National Center for Biotechnology Information (NCBI) under the BioProject accession number PRJNA748851.

Multilocus sequence typing (MLST) and core genome (cg)MLST Analysis

Genome assemblies produced in our study, along with 67 public genomes available at GenBank (accessed on 28 October 2020), were genotyped using cgMLST. The cgMLST profiles were assigned using the task Template *B. melitensis/suis/canis/abortus* cgMLST with 2076 targets core genes in Ridom SeqSphere+ software, v4.1.1 (Ridom GmbH, Münster, Germany) as described by Janowicz and colleagues (Janowicz *et al.* 2018). Multiple spanning tree (MST) was generated by pairwise comparison of cgMLST target genes using default parameters. The Neighbour-Joining Tree was constructed by the distance allele table and circular tree was visualized using iTol (Letunic and Bork 2019). Missing values were ignored in the calculation of distance between pairs of sample profiles. All sequences were additionally typed using the *Brucella* 9 locus MLST scheme available at https://pubmlst.org/brucella/ (Whatmore *et al.* 2007) accessible through Ridom SeqSphere+.

Statistical analysis

Data from Laboratory Information Management System were imported in MS Access® (Microsoft Access 2019, Redmond, Washington, DC, USA), which was used for cleaning and normalizing the dataset. To take into account the uncertainty of the proportion of positive laboratory results over the total tests performed, a beta distribution was used to define the 95% confidence interval of the proportion accuracy. The uncertainty interval was defined as the difference between upper and lower 95% confidence limits. The 95% lower and upper credibility levels (L.C.I. and U.C.L., respectively, composing the Credibility Interval, CI) of the distribution frequency of positive results were calculated using a Bayesian approach (Sivia 1996) with a beta distribution (n + 1; n - s + 1), where n is the total number of tested samples and s are the tested positive samples.

Results

Population data

The total number of dogs considered in this study was 683. Out of them, 475 (69.5%) were females and 208 (30.5%) were males. The distribution of dogs by age at the time of sampling is shown in Figure 1. The dogs in the kennel were 2.8 years old in average, with the oldest dog being 8.5 year old. The most represented breed was Chihuahua (605 dogs, 88.6%), followed by Spitz (37 dogs, 5.4%),

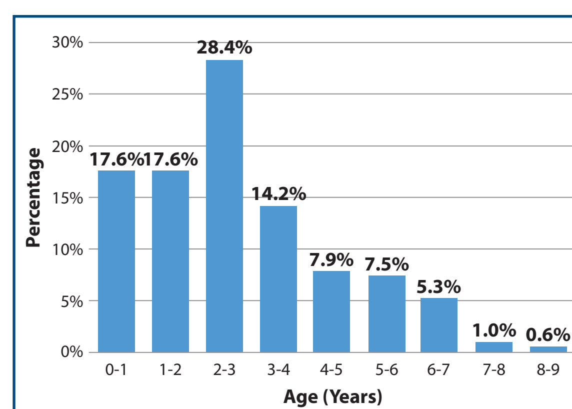

Figure 1. *Age distribution of dogs at the time of sampling.*

Poodle (29 dogs, 4.2%), Maltese (7 dogs, 1.0%), Pug (3 dogs, 0.4%), Tibetan mastiff (1 dog, 0.1%), and a cross-breed dog (1 dog, 0.1%).

Serology

In the first round of sampling, 269 tested positive out of 598 animals sampled. Given that 15 sera were not analysed because haemolytic, the apparent seroprevalence was 46.1%. In the second round of sampling, out of the 683 serum samples collected, 241 were positive, for an apparent prevalence of 35.3%. Among the 241 seropositive animals, 64 were also positive to blood culture (26.6%). In four seronegative animals, *B. canis* was isolated from blood cultures (Table I). When analysing the performances of the two tests, the Cohen's Kappa value obtained was 0.307, considered as fair in the interpretation of strength of agreement suggested by Landis and Kock (Landis and Kock 1977).

Comparing the results of serological testing during the first and second round of sampling, 220 animals remained serologically positive, 45 animals became negative, while 15 animals became seropositive. When considering the same group of animals for the first and second round of sampling, seroprevalence decreased from 46.1% to 40.0%.

We also calculated the specificity of mSAT on 143 sera from owned dogs not-related to the outbreak. mSAT identified 138 samples as negative, demonstrating a specificity of 96.5% (CI 92.1%-98.5%).

The distribution of seropositivity was different between males and females. Overall, 48 males were positive out of 208 (23.1%), while 193 females were positive out of 475 (40.6%). This difference was found as significant ($\chi^2 = 19.52$; $p < 0.01$).

When age classes were considered, the distribution of seropositivity was higher in the classes from 2 to 7 years. Lower rates of seropositivity were recorded above and under that age interval (Table II). When considering the credibility intervals, the number of seropositives under one year of age was significantly lower than the other age classes.

The distribution of seropositivity according to breed is shown in Table III. The seropositivity in Poodle breed was significantly higher than the seropositivity recorded for Chihuahua breed ($\chi^2 = 9.38$; $p < 0.01$).

Brucella isolation and identification

Out of the 683 whole blood samples collected, 68 were positive for microbiological isolation. All strains isolated were confirmed as *B. canis* by PCR assay. Moreover, 61 samples grew on direct sowing, while seven strains were detected only by subcultures from enrichment broth. Furthermore, real-time PCR allowed the identification of 32 positive dogs. Results are summarized in Table IV. When analysing the performances of the two tests, the Cohen's Kappa value obtained was 0.381,

Table I. *Comparison of positive and negative results obtained by* B. canis *microplate serum agglutination test (mSAT) and* Brucella *spp. isolation method.*

		Serology (mSAT)		
		Positive	**Negative**	**Total**
Isolation	Positive	64	4	**68**
	Negative	177	438	**615**
	Total	**241**	**442**	**683**

Table II. *Distribution of seropositivity by age classes.*

Age class	N. tested	N. positive	% positive	L.C.L.	U.C.L.
0 - 1	120	5	4.2%	1.8%	9.4%
1 - 2	120	40	33.3%	25.5%	42.2%
2 - 3	194	87	44.8%	38.0%	51.9%
3 - 4	97	43	44.3%	34.8%	54.3%
4 - 5	54	26	48.1%	35.4%	61.2%
5 - 6	51	22	43.1%	30.5%	56.8%
6 - 7	36	15	41.7%	27.1%	57.9%
7 - 8	7	2	28.6%	8.5%	65.1%
8 - 9	4	1	25.0%	5.3%	71.6%
Total	**683**	**241**	**35.3%**	**31.8%**	**38.9%**

Table III. *Distribution of seropositivity by breed.*

Breed	N. tested	N. positive	% positive	L.C.L.	U.C.L.
Chihuahua	605	207	34.2%	30.5%	38.1%
Spitz	37	13	35.1%	21.8%	51.4%
Poodle	29	18	62.1%	43.9%	77.3%
Maltese	7	1	14.3%	3.2%	52.7%
Pug	3	2	66.7%	19.4%	93.2%
Cross-Breed	1	0	0.0%	1.3%	84.2%
Tibetan Mastiff	1	0	0.0%	1.3%	84.2%
Total	**683**	**241**	**35.3%**	**31.8%**	**38.9%**

Table IV. *Comparison of positive and negative results obtained on EDTA blood samples by isolation and real-time PCR for* B. canis *identification.*

		Real-time PCR		
		Positive	**Negative**	**Total**
Isolation	Positive	21	47	**68**
	Negative	11	604	**615**
	Total	**32**	**651**	**683**

Table V. *Distribution of dogs positive to* B. canis *isolation according to age classes.*

Age class	N. tested	N. positive	% positive	L.C.L.	U.C.L.
0 - 1	120	6	5.0%	2.4%	10.5%
1 - 2	120	13	10.8%	6.5%	17.7%
2 - 3	194	26	13.4%	9.3%	18.9%
3 - 4	97	11	11.3%	6.5%	19.2%
4 - 5	54	3	5.6%	2.0%	15.1%
5 - 6	51	5	9.8%	4.4%	21.0%
6 - 7	36	3	8.3%	3.0%	21.9%
7 - 8	7	1	14.3%	3.2%	52.7%
8 - 9	4	0	0.0%	0.0%	45.1%
Total	**683**	**68**			

considered as fair in the interpretation of strength of agreement suggested by Landis and Kock (Landis and Kock 1977).

The distribution of dogs positive to *B. canis* isolation was different between males and females. Overall, 13 males were positive out of 208 (6.3%), while 55 females were positive out of 475 (11.6%). This difference was found as significant ($\chi^2 = 4.58$; $p < 0.05$). When age classes were considered, the percentage of dogs positive to bacteriology was higher in the classes from 2 to 3 years, and from 7 to 8 years (Table V). However, when considering the credibility intervals, no significant difference was recorded between age classes.

The distribution of positivity to isolation according to breed is shown in Table VI. The rate of isolation in Poodle breed was significantly higher than the rate recorded for Chihuahua breed ($\chi^2 = 19.92$; $p < 0.01$)

Genomic analysis

Genome assemblies were used to retrieve MLST profiles, which identified for all the strains analysed the Sequence Type (ST) 21. The assignation of the cgMLST profiles comprising 2074 targets retrieved at least 98.7% of them for all the strain analysed. The cluster analysis from the outbreak revealed that all the strains belong to the same cluster (Figure 2). The maximum distance observed between strains isolated in the outbreak was four allelic differences, thus confirming the hypothesis that the outbreak has been generated by a single introduction of *B. canis* in the kennel.

The comparison of the cgMLST profiles against the public genomes revealed that the *B. canis* population was divided in two main groups corresponding to ST20, which was previously linked to South, Central and North America, and ST21 mostly linked to Asia and Europe (Vicente *et al.* 2018). The outbreak

Table VI. *Distribution of dogs positive to* B. canis *isolation by breed.*

Breed	N. tested	N. positive	% positive	L.C.L.	U.C.L.
Chihuahua	605	54	8.9%	6.9%	11.5%
Spitz	37	4	10.8%	4.4%	24.8%
Poodle	29	10	34.5%	19.9%	52.8%
Maltese	7	0	0.0%	0.3%	36.9%
Pug	3	0	0.0%	0.6%	60.2%
Cross-Breed	1	0	0.0%	1.3%	84.2%
Tibetan Mastiff	1	0	0.0%	1.3%	84.2%
Total	**683**	**68**	**10.0%**	**7.9%**	**12.4%**

strains fell in the ST21 group, identifying that closest genome was isolated in China on an unspecified date (Figure 3).

Discussion and conclusions

This is the first report of the isolation of *B. canis* in dogs in Italy, as well as the first report of the occurrence of an outbreak in an Italian commercial breeding kennel. Moreover, and to the best of our knowledge, this is also the first report worldwide of an outbreak of *B. canis* infection involving very high number of dogs.

Until the bacterial isolation described in this study, brucellosis due to *B. canis* was considered a foreign disease for Italy. Previous recordings were based on serological evidence only, with the exception of a single report of *B. canis* identification by PCR, in a dog with chronic prostatitis and discospondylitis (Corrente *et al.* 2010). Private veterinarians or

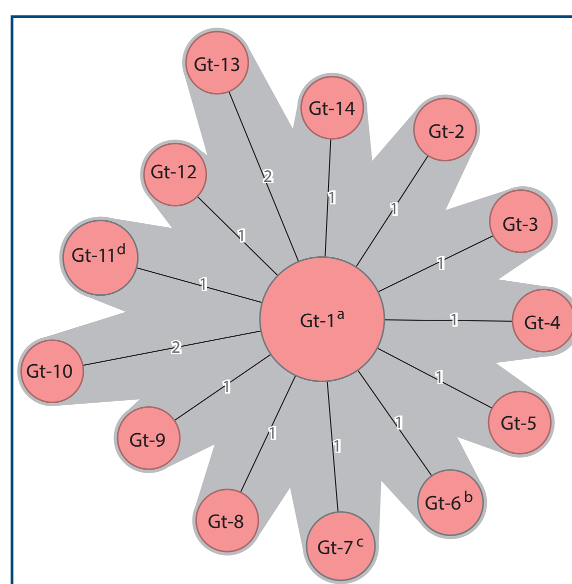

Figure 2. *Minimum spanning tree generated for 67 dog isolates.* Each circle represents an allelic profile. The numbers on the connecting lines illustrate the numbers of target genes with differing alleles.

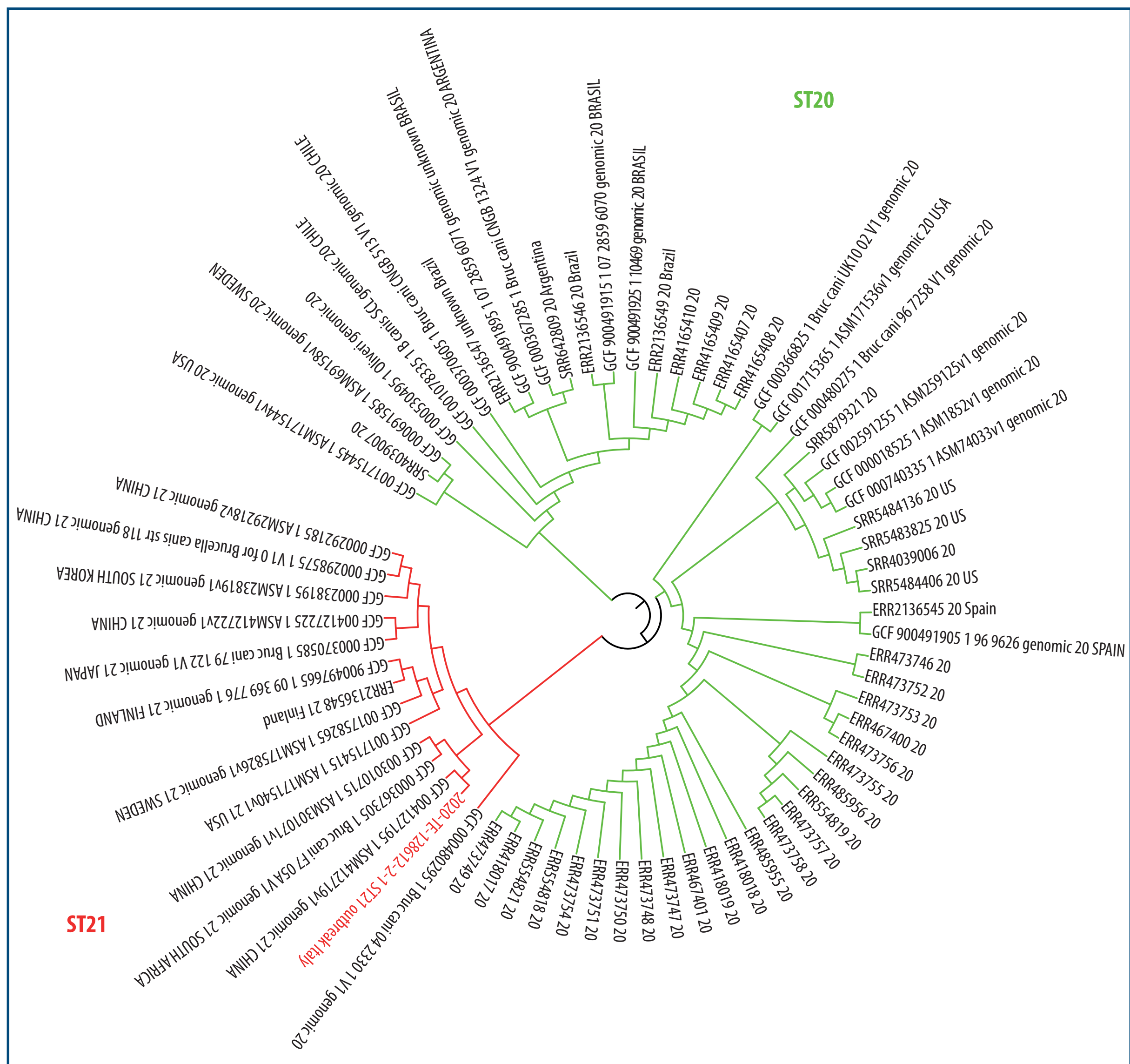

Figure 3. *Neighbour-Joining tree of* B. canis. The tree was constructed using distance allele table sequences of 68 isolates and mid-point rooted. The label from the outbreak clone is reported in red. Sequence type groups are reported in green for ST20 and red for ST21.

public health authorities have rarely investigated this zoonosis, also due to the lack of regulations on canine brucellosis surveillance or provisions for specific controls in dog international trade for this disease. This contributed to the current lack of data on *B. canis* diffusion among the Italian dog population, and to the limited knowledge about the disease distribution in the European Union (EU) canine population. Furthermore, considering its zoonotic potential, uncontrolled spread of *B. canis* may have important public health implications.

In Italy, the veterinary regulations do not provide specific restrictive measures for canine brucellosis caused by *B. canis*. Actually, brucellosis and agents thereof are included in the list of the zoonosis requiring surveillance on Annex I of the so-called "Zoonoses Directive" (EU 2003). However, in the context of canine brucellosis, current rules for brucellosis control and eradication in ruminants would not be acceptable for application in dogs, both for animal welfare and ethical reasons. On the other hand, due to possible health implications, either for dogs and their owners, actions are required to acquire data on distribution of this underhanded zoonosis of pets, as well as for controlling his spread in situations where it occurs. Thus, protocols are necessary for proper detection, control, and eradication of the infection in kennels and in owned dogs.

Laboratory tests are a cornerstone for *B. canis* diagnosis and control. Serological testing represents a valid screening tool for evaluating the presence of *B. canis* in breeding kennels. Based on serological results, apparent seroprevalence during the first round of sampling (598 animals) was 46.1% while in the second round of sampling (683 animals)

decreased to 35.3%. These data resemble or even exceed the highest values reported in the literature (Hensel *et al.* 2018), giving a clear number of the impact the disease may have in a kennel. The high seroprevalence is probably related and consequent to the high density dog population hosted in the kennel, in addition to inadequate kennel management, both factors contributing to *B. canis* spreading. We observed a reduced apparent seroprevalence between the first and second sampling. This result is partially explained with the increased number of animal tested during the second sampling. Actually, the additional population tested in second sampling was composed by young animals, and most of them resulted seronegative. Someone may also argue that the decreased prevalence observed was related to the antibody isotype switching that does occur as the disease progresses, when the most prevalent *B. canis* specific antibodies found in serum after infection shift from IgMs to IgGs. IgGs are characterised by lower agglutination properties compared to IgMs. In the hypothesis that mSAT mainly detect IgMs, mSAT would have a reduced sensitivity on sera with high concentrations of *B. canis* specific IgGs but low IgMs. In our opinion, this was not the case and previous studies in human, performed on similar test, showed that even when IgMs are removed, serum agglutination capacity, despite reduced, was maintained by IgG and IgA antibodies (Marrodan *et al.* 2001). This suggests that also animals with lower amount of IgMs compared to IgGs should test positive to mSAT. On the other hand, these observations support the use in parallel of serological tests that evaluate IgG antibodies, in addition to IgMs, this in order to maximize the chance of detecting seropositive animals. Finally, the non-optimal specificity observed for mSAT may have influenced the different prevalence observed between the first and second sampling. In fact, 32 animals that tested positive to mSAT during the first sampling, with an antibody titer around the cut-off value, turned negative few weeks later. A fair agreement was recorded between isolation and mSAT testing procedures (Cohen's Kappa value 0.307). During the outbreak investigation, we identified four cases of active infection and bacteraemia that tested negative to serological tests. As previously described (Carmichael 1990, Carmichael and Greene 2006) antibody response only appears 5-8 weeks after infection and animals with ongoing bacteraemia may result negative to serological tests for 3-4 weeks post infection. Similarly, animals with chronic infection may also result negative to serological screening (Carmichael and Greene 2006).

The study also evaluated the disease prevalence among gender and age. The rate of positivity to laboratory tests was significantly higher in females than in males, both for serology and isolation tests. Actually, in a free-living dog population and in a situation of uncontrolled spread of the disease, it would be expected to have a similar prevalence in both genders. However, giving that the outbreak occurred in a commercial breeding kennel, this finding could be the result of breeding practices. In fact, one hypothesis is that to maintain a good bloodline not all males were allowed to mate and then only a fraction of them was actually exposed to the infection from the venereal route.

The rate of positivity was distributed evenly across age classes, except for dogs aged one year or under. This is suggestive of an infection that remained uncontrolled for a long period of time, favouring the disease spread among dogs of all age classes, both through mating and environmental contamination. Similar observations on the low susceptibility of animals under the age of reproduction to brucellosis were also reported in other animal species (Nicoletti 1980). However, it is not clear if this resistance or lower susceptibility of young animals is related to immature reproductive system, a reduced time of exposure to infection, or other factors that remain to be investigated.

When looking at the breed involved in the *B. canis* outbreak, positivity was found significantly higher in Poodles, even if this breed was low in numbers compared to Chihuahuas. This may suggest that the infection was introduced in the kennel with this breed and then was transmitted to other dogs due to environmental contamination after abortion.

The fair agreement recorded between isolation and PCR testing procedures (Cohen's Kappa value 0.381), considering that the isolation gave positive results in a higher number of specimens with respect to PCR, suggests that the PCR assay has difficulties in performing on blood matrix. In the literature the sensitivity of PCR for *Brucella* is very variable when performed on blood matrix, ranging from 3.26% (Kauffman *et al.* 2012) to 97.14% (Keid *et al.* 2010). This may be due to the presence of PCR-inhibitory molecules like haemoglobin (Sidstedt *et al.* 2018), which may affect the amplification process through different mechanisms or lead to a failure of amplicons detection. Not only the presence of inhibitors, but also the use of antibiotics and heparin blood sampling could alter the sensitivity of the PCR results (Mol *et al.* 2020). To improve the performance of the method, it is possible to proceed with centrifugation of the blood sample and extraction of the DNA from buffy coat. However, larger quantities of blood are required for this purpose, and often they are not available from dogs due to the breed size. Also because *Brucella* is a facultative intracellular bacterium, the DNA yield during extraction may be low. This critical point may be solved by sowing

in enrichment broth and extracting DNA from the one-week incubation culture.

The genomic analysis of strains isolated from the outbreak revealed that they all belong to the same cluster (Figure 1). The maximum distance observed between strains isolated in the outbreak was only of four allelic differences, thus confirming the hypothesis that the outbreak under study has been generated by introduction of a single of *B. canis* strain in the kennel. The number of allelic differences was within the published cut-off of genomic cluster found out in a *B. melitensis* outbreak (Janowicz *et al.* 2018). Moreover, these limits were also reasonable if compared with several bovine brucellosis outbreaks. These observations strengthen the idea that a single *B. canis* introduction led to unrestrained spread within the kennel, affecting many dogs and puppies. Importantly, by surveilling *B. canis* sequences from the worldwide database, it was not possible to trace back this strain; however, the availability of the genomes generated by the present study will allow the trace forward and the surveillance of the infection spread from this outbreak. This study highlights the need of further data for an updated genomic surveillance with the aim to avoid public health consequences related to a poor mitigation strategy in fighting the spread of this disease at national and international level.

The scarce information about *B. canis* contained in the international *Brucella* genomic databases has made not possible to give further details on the genomic differences of the strains isolated, which would have been useful to trace back the infection source. Indeed, to better help epidemiological investigations in *B. canis* outbreaks, it would be needed to genotype as much *B. canis* isolates as possible worldwide, in order to improve the quantity and quality of information stored in the international genomic databases.

Further circumstances prevented the identification of the source of infection in the kennel under study. Dogs were kept in a commercial breeding kennel, where epidemiological investigation on movements of breeding dogs were not conclusive. Furthermore, from the occurrence of abortions to the diagnosis of canine brucellosis several months elapsed, allowing the infection spreading largely in the kennel, thus hampering the possibility to identify the origin of the infection and, therefore, to trace back its source.

Public and private veterinarians should be trained in canine brucellosis diagnosis, prevention and control, and should investigate the causes of abortion in dogs considering also *B. canis*. More research on the proper use of antimicrobials in affected dogs is advisable, at least until a reliable vaccine will become available. This highlights also the importance of reminding breeders of the clinical signs of canine brucellosis and their responsibility to prevent intra-population and inter-population spread of the disease and possible human infections. Surviving puppies can be an important source of *B. canis* infection, as they can become permanent carriers and shedders of the pathogen. All cases of canine abortion should be examined for brucellosis by bacterial culture of the foetuses and placentas. Commercial breeding kennels should be regularly checked for causes of abortion and international trade rules should foresee testing for *B. canis* of imported breeding dogs.

Finally, more researches are required in order to enhance the performances of the diagnostic tests in terms of sensitivity and specificity, as well as more survey data are needed to determine the current spread of *B. canis* in Italy, in the light to identify the related epidemiological patterns and burden of the disease on Italian canine populations, as well as on public health.

References

Alton G.G., Jones L.M., Angus R.D. & Verger J.M. 1988. Techniques for the brucellosis laboratory. I.N.R.A., Paris, 169-174.

Auwera G.A., Carneiro M.O., Hartl C., Poplin R., Levy-Moonshine A., Jordan T., Shakir K., Roazen D., Thibault J., Banks E., Garimella K.V., Altshuler D., Gabriel S. & DePristo M.A. 2013. From FastQ data to high-confidence variant calls: the genome analysis toolkit best practices pipeline. *Curr Protoc Bioinformatics*, **43**, 11.10.1-11.10.33.

Bankevich A., Nurk S., Antipov D., Gurevich A.A., Dvorkin M., Kulikov A.S., Lesin V.M., Nikolenko S.I., Pham S., Prjibelski A.D., Pyshkin A.V., Sirotkin A.V., Vyahhi N., Tesler G., Alekseyev M.A. & Pevzner P.A. 2012. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *J Comput Biol*, **19**, 455-477. https://doi.org/10.1089/cmb.2012.0021.

Blankenship R.M. & Sanford J.P. 1975. *Brucella canis*: a cause of undulant fever. *Am J Med*, **59**, 424-426.

Brower A., Okwumabua O., Massengill C., Muenks Q., Vanderloo P., Duster M., Homb K. & Kurth K. 2007. Investigation of the spread of *Brucella canis* via the U.S. interstate dog trade. *Int J Infect Dis*, **11**, 454-458. doi: 10.1016/j.ijid.2006.12.009.

Cadmus S.I.B., Adesokan H.K., Ajala O.O., Odetokun W.O., Perrett L.L. & Stack J.A. 2011. Seroprevalence of *Brucella abortus* and *B. canis* in household dogs in southwestern Nigeria: a preliminary report. *J S Afr Vet Assoc*, **82**, 56-57.

Carmichael L.E., Zoha S.J. & Flores-Castro R. 1984. Problems in the serodiagnosis of canine brucellosis: dog responses to cell wall and internal antigens of *Brucella canis*. *Dev Biol Stand*, **56**, 371-383.

Carmichael L.E. & Greene G.E. 2013. Canine brucellosis. *In* Infectious diseases of the dog and cat (Greene G.E., ed.). 4 ed. Saunders, Philadelphia, 398-410.

Carmichael L.E. & Shin S.J. 1996. Canine brucellosis: a diagnostician's dilemma. *Semin Vet Med Surg (Small Anim)*, **11**, 161-165.

Carmichael L.E. 1966. Abortion in 200 beagles. *J Am Vet Med Assoc*, **149**, 1126.

Carmichael L.E. 1990. *Brucella canis. In* Animal Brucellosis (Nielsen K. & Duncan J.R., eds). CRC Press, Boca Raton, Florida, 335-350.

Carmichael L.E., Flores-Castro R. & Zoha S. 1980. Brucellosis caused by *Brucella canis*: an update of infection in animals and in humans. Geneva, World Health Organization. Document WHO/BRUC./80.361 WHO/ZOON./80.135.

Carriço J.A., Silva-Costa C., Melo-Cristino J., Pinto F.R., de Lencastre H., Almeida J.S. & Ramirez M. 2006. Illustration of a common framework for relating multiple typing methods by application to macrolide-resistant *Streptococcus* pyogenes. *J Clin Microbiol*, **44**, 2524-2532.

Center for Food Security and Public Health (CFSPH). 2020. The canine brucellosis. *Brucella canis*. Ames, Iowa. http://www.cfsph.iastate.edu/Factsheets/pdfs/brucellosis_canis.pdf.

Chinyoka S., Dhliwayo S., Marabini L., Dutlow K., Matope G. & Pfukenyi D.M. 2014. Serological survey of *Brucella canis* in dogs in urban Harare and selected rural communities in Zimbabwe. *J S Afr Vet Assoc*, **85**, 1087.

Corrente M., Franchini D., Decaro N., Greco G., D'Abramo M., Greco M.F., Latronico F., Crovace A. & Martella V. 2010. Detection of *Brucella canis* in a dog in Italy. *New Microbiol*, **33**, 337-341.

Cosford K.L. 2018. *Brucella canis*: an update on research and clinical management. *Can Vet J*, **59**, 74-81.

Daly R., Willis K.C., Wood J., Brown K., Brown D., Beguin-Strong T., Smith R. & Ruesch H. 2020. Seroprevalence of *Brucella canis* in dogs rescued from South Dakota Indian reservations, 2015-2019. *Prev Vet Med*, **184**, 105157.

Di D., Cui B., Wang H., Zhao H., Piao D., Tian L., Tian G., Kang J., Mao X., Zhang X., Du P., Zhu L., Zhao Z., Mao L., Yao W., Guan P., Fan W. & Jiang X. 2014. Genetic polymorphism characteristics of *Brucella canis* isolated in China. *PLoS ONE*, **9**, 1-7.

Dreer M.K.P., Dib Gonçalves D.D., Caetano I.C., Gerônimo E., Menegas P.H., Bergo D., Lopes-Mori F.M.R., Benitez A., de Freitas J.C., Evers F., Navarro T.I. & de Almeida Martins L. 2013. Toxoplasmosis, leptospirosis, and brucellosis in stray dogs housed at the shelter in Umuarama municipality, Paraná, Brazil. *J Venom Anim Toxins Incl Trop Dis*, **19**, 23.

Egloff S., Schneeberger M., Gobeli Brawand S., Krudewig C., Schmitt S., Reichler I. & Peterhans S. 2018. *Brucella canis* infection in a young dog with epididymitis and orchitis. *Schweiz Arch Tierheilkd*, **160** (12), 743-748.

European Union (EU). 2003. Directive 2003/99/EC of the European Parliament and of the Council of 17 November 2003 on the monitoring of zoonoses and zoonotic agents, amending Council Decision 90/424/EEC and repealing Council Directive 92/117/EEC. *OJ*, **L 325**, 12/12/2003.

Flores-Castro R., Suarez F., Ramirez-Pfeiffer C. & Carmichael L.E. 1977. Canine brucellosis: bacteriological and serological investigation of naturally infected dogs in Mexico City. *J Clin Microbiol*, **6**, 591-597.

Gardner D. & Reichel M. 1997. No evidence of *Brucella canis* infection in New Zealand dogs. *Surveillance*, **24**, 17-18.

Garofolo G., Ancora M. & Di Giannatale E. 2013. MLVA-16 loci panel on *Brucella* spp. using multiplex PCR and multicolor capillary electrophoresis. *J Microbiol Methods*, **92**, 103-107.

George L.W., Duncan J.R. & Carmichael L.E. 1979. Semen examination in dogs with canine brucellosis. *Am J Vet Res*, **40** (11), 1589-1595.

Georgia Department of Agriculture (GDA). 2020. Canine Brucellosis (*Brucella canis*). Atlanta, Georgia. http://agr.georgia.gov/Data/Sites/1/media/ag_animalindustry/animal_health/files/caninebrucellosis.pdf.

Gyuranecz M., Szeredi L., Rónai Z., Dénes B., Dencso L., Dán Á., Pálmai N., Hauser Z., Lami E., Makrai L., Erdélyi K. & Jánosi S. 2011. Detection of *Brucella canis*-induced reproductive diseases in a Kennel. *J Vet Diagnostic Investig*, **23**, 143-147.

Hayashi T.T.A & Ysayama Y. 1977. Detection of *Brucella canis* infection in dogs in Hokkaido. *Microbiol Immunol*, **21** (5), 295-298.

Hensel M.E., Negron M. & Arenas-Gamboa A.M. 2018. Brucellosis in dogs and public health risk. *Emerg Infect Dis*, **24**, 1401-1406.

Hofer E., Bagó Z., Revilla-Fernández S., Melzer F., Tomaso H., López-goñi I., Fasching G. & Schmoll F. 2012. First detection of *Brucella canis* infections in a breeding kennel in Austria. *New Microbiol*, **35**, 507-510.

Holst B.S., Löfqvist K., Ernholm L., Eld K., Cedersmyg M. & Hallgren G. 2012. The first case of *Brucella canis* in Sweden: background, case report and recommendations from a northern European perspective. *Acta Vet Scand*, **54**, 18.

Janowicz A., De Massis F., Ancora M., Cammà C., Patavino C., Battisti A., Prior K., Harmsen D., Scholz H., Zilli K., Sacchini L., Di Giannatale E. & Garofolo G. 2018. Core genome multilocus sequence typing and single nucleotide polymorphism analysis in the epidemiology of *Brucella melitensis* infections. *J Clin Microbiol*, **56** (9), e00517-18.

Junemann S., Sedlazeck F.J., Prior K., Albersmeier A., John U., Kalinowski J., Mellmann A., Goesmann A., von Haeseler A., Stoye J. & Harmsen D. 2013. Updating benchtop sequencing performance comparison. *Nat Biotechnol*, **31**, 294-296.

Kaden R., Ågren J., Båverud V., Hallgren G., Ferrari S., Börjesson J., Lindberg M., Bäckman S. & Wahab T. 2014. Brucellosis outbreak in a Swedish kennel in 2013: determination of genetic markers for source tracing. *Vet Microbiol*, **174**, 523-530.

Kauffman L.K., Bjork J.K., Gallup J.M., Boggiatto P.M., Bellaire B.H. & Petersen C.A. 2014. Early detection of *Brucella canis* via quantitative polymerase chain reaction analysis. *Zoonoses Public Health*, **61**, 48-54.

Kaufmann L.K. & Petersen C.A. 2019. Canine brucellosis: old foe and reemerging scourge. *Vet Clin North Am Small Anim Pract*, **49** (4), 763-779.

Keid L.B., Soares R.M., Vasconcellos S.A., Salgado V.R., Megid J. & Richtzenhain L.J. 2010. Comparison of a PCR assay in whole blood and serum specimens for canine brucellosis diagnosis. *Vet Rec*, **167**, 96-99.

Keid L.B., Chiebao D.P., Batinga M.C.A., Faita T., Diniz J.A., Oliveira T.M.F. de S., Ferreira H.L. & Soares R.M. 2017. *Brucella canis* infection in dogs from commercial breeding kennels in Brazil. *Transbound Emerg Dis*, **64**, 691-697.

Keid L.B., Soares R.M., Vasconcellos S.A., Megid J., Salgado V.R. & Richtzenhain L.J. 2009. Comparison of agar gel immunodiffusion test, rapid slide agglutination test, microbiological culture and PCR for the diagnosis of canine brucellosis. *Res Vet Sci*, **86**, 22-26.

Krueger W.S., Lucero N.E., Brower A., Heil G.L. & Gray G.C. 2014. Evidence for unapparent *Brucella canis* infections among adults with occupational exposure to dogs. *Zoonoses Public Health*, **61**, 509-518.

Landis J.R. & Koch G.G. 1977. The measurement of observer agreement for categorical data. *Biometrics*, **33**, 159-174.

Le Flèche P., Jacques I., Grayon M., Al Dahouk S., Bouchon P., Denoeud F., Nockler K., Neubauer H., Guilloteau L.A. & Vergnaud G. 2006. Evaluation and selection of tandem repeat loci for a *Brucella* MLVA typing assay. *BMC Microbiol*, **6**, 9.

Letunic I. & Bork P. 2019. Interactive tree of life (iTOL) v4: recent updates and new developments. *Nucleic Acids Res*, **47**(W1), W256-W259.

Li H. 2013. Aligning sequence reads, clone sequences, and assembly contigs with BWA-MEM. *arXiv*, 13033997v1. https://arxiv.org/abs/1303.3997.

Marrodan T., Nenova-Poliakova R., Rubio M., Ariza J., Clavijo E., Smits H.L. & Diaz R. 2001. Evaluation of three methods to measure anti-*Brucella* IgM antibodies and interference of IgA in the interpretation of mercaptan-based tests. *J Med Microbiol*, **50** (8), 663-666.

Mellmann A., Harmsen D., Cummings C.A., Zentz E.B., Leopold S.R., Rico A., Prior K., Szczepanowski R., Ji Y., Zhang W., McLaughlin S.F., Henkhaus J.K., Leopold B., Bielaszewska M., Prager R., Brzoska P.M., Moore R.L., Guenther S., Rothberg J.M. & Karch H. 2011. Prospective genomic characterization of the German enterohemorrhagic *Escherichia coli* O104:H4 outbreak by rapid next generation sequencing technology. *PLoS One*, **6**, e22751.

Michaux-Charachon S., Jumas-Bilak E., Allardetservent A., Bourg G., Boschirolim L. & O'Callaghan D. 2002. The *Brucella* genome at the beginning of the post-genomic era. *Vet Microbiol*, **90**, 581-585.

Mol J.P.S., Guedes A.C.B., Eckstein C., Quintal A.P.N., Souza T.D., Mathias L.A., Haddad J.P.A., Paixao T.A. & Santos R.L. 2020. Diagnosis of canine brucellosis: comparison of various serologic tests and PCR. *J Vet Diagn Invest*, **32**, 77-86.

Mor S.M., Wiethoelter A.K., Lee A., Moloney B., James D.R. & Malik R. 2016. Emergence of *Brucella suis* in dogs in New South Wales, Australia: clinical findings and implications for zoonotic transmission. *BMC Vet Res*, **12**, 199.

Nascimento M., Sousa A., Ramirez M., Francisco A.P., Carrico J.A. & Vaz C. 2017. PHYLOViZ 2.0: providing scalable data integration and visualization for multiple phylogenetic inference methods. *Bioinformatics*, **33**, 128-129.

National Association of State Public Health Veterinarians (NASPHV). 2012. Public health implications of *Brucella canis* infections in humans. http://nasphv.org/Documents/BrucellaCanisInHumans.pdf.

Nicoletti P. 1980. The epidemiology of bovine brucellosis. *Adv Vet Sci Comp Med*, **24**, 69-98.

Nöckler K., Kutzer P., Reif S., Rosenberger N., Draeger A., Bahn P., Göllner C. & Erlbeck C. 2003. Canine brucellosis - a case report. *Berl Munch Tierarztl Wochenschr*, **116**, 368-372.

Nurk S., Bankevich A., Antipov D., Gurevich A.A., Korobeynikov A., Lapidus A., Prjibelski A.D., Pyshkin A., Sirotkin A., Sirotkin Y., Stepanauskas R., Clingenpeel S.R.,

Woyke T., McLean J.S., Lasken R., Tesler G., Alekseyev M.A. & Pevzner P.A. 2013. Assembling single-cell genomes and minimetagenomes from chimeric MDA products. *J Comput Biol*, **20**, 714-737.

Rovid Spickler A. 2018. Brucellosis: *Brucella canis*. United States Department of Agriculture Animal and Plant Health Inspection Service, 1-10. http://www.cfsph.iastate.edu/Factsheets/pdfs/brucellosis_canis.pdf.

Sahl J.W., Beckstrom-Sternberg S.M., Babic-Sternberg J., Gillece J.D., Hepp C.M., Auerbach R.K., Tembe W., Wagner D.M., Keim P.S. & Pearson T. 2015. The *in silico* genotyper (ISG): an open-source pipeline to rapidly identify and annotate nucleotide variants for comparative genomics applications. *bioRxiv*, 1-7. https://doi.org/10.1101/015578.

Severiano A., Pinto F.R., Ramirez M. & Carriço J.A. 2011. Adjusted Wallace coefficient as a measure of congruence between typing methods. *J Clin Microbiol*, **49**, 3997-4000.

Shin S. & Carmichael L.E. 1999. Canine brucellosis caused by *Brucella canis. In* Recent advances in canine infectious disease (Carmichael L.E., ed). New York International Veterinary Information Service, Ithaca NY, 1-4.

Sidstedt M., Hedman J., Romsos E.L., Waitara L., Wadsö L., Steffen C.R., Vallone P.M. & Rådström P. 2018. Inhibition mechanisms of hemoglobin, immunoglobulin G, and whole blood in digital and real-time PCR. *Anal Bioanal Chem*, **410**, 2569-2583.

Sivia D.S. 1996. Data Analysis: a bayesian Tutorial. Oxford University Press, Oxford, UK.

Taylor D.J. 1980. Serological evidence for the presence of *Brucella canis* infection in dogs in Britain. *Vet Rec*, **106**, 102-104.

United States Department of Agriculture Animal and Plant Health Inspection Service (USDA). 2015. Best practices for *Brucella canis* prevention and control in dog breeding facilities. U.S. Department of Agriculture. https://www.aphis.usda.gov/animal_welfare/downloads/brucella_canis_prevention.pdf.

Vicente A.F., Girault G., Corde Y., Mioni M.S.R., Keid L.B., Jay M., Megid J. & Mick V. 2018. New insights into phylogeography of worldwide *Brucella canis* isolates by comparative genomics-based approaches: focus on Brazil. *BMC Genomics*, **19** (1), 636.

Von Kruedener R.B. 1976. Outbreak of a *Brucella canis* infection in a beagle colony in West Germany. *Dev Biol Stand*, **31**, 251-253.

Wanke M.M. 2004. Canine brucellosis. *Anim Reprod Sci*, **82-83**, 195-207.

Whatmore A., Perret L. & Friggens M. 2017. Second UK isolation of *Brucella canis. Vet Rec*, **180** (25), 617.

Whatmore A.M., Perrett L.L. & MacMillan A.P. 2007. Characterisation of the genetic diversity of *Brucella* by multilocus sequencing. *BMC Microbiol*, **7**, 34.

World Organization for Animal Health (OIE). 2019. Brucellosis (*Brucella abortus, B. melitensis* and *B. suis*) (infection with *B. abortus, B. melitensis* and *B. suis*) *In* Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. Paris, Office International des Epizooties, 1-44.

Yoak A.J., Reece J.F., Gehrt S.D. & Hamilton I.M. 2014. Disease control through fertility control: secondary benefits of animal birth control in Indian street dogs. *Prev Vet Med*, **113**, 152-156.