Genetic characterization of Brucella melitensis *and* Brucella abortus *geographical clusters in Italy*

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Keywords
Brucella abortus,
Brucella melitensis,
Genotyping,
Italy,
MLVA,
Ruminants.

Summary
The genetic diversity of *Brucella melitensis* and *Brucella abortus* strains isolated in 199 cattle and sheep from 156 brucellosis outbreaks which occurred in 8 regions of Southern Italy in 2011, was determined using a Multiple-Locus Variable Number of Tandem Repeats Analysis (MLVA-16) approach. The existence of possible genetic clusters was verified through a hierarchical cluster analysis based on 'single link', which is closely related to the minimum spanning tree. The Hamming weighted distance matrix was adopted in the analysis. All calculations were performed using R and the additional libraries phangorn and Cluster. For a number of clusters, ranging from 2 to 15, the average silhouette width was calculated. The number of clusters adopted was identified according to the maximum average silhouette width. For *B. abortus* and *B. melitensis*, 6 and 11 genetic clusters were identified, respectively. Three out of 6 *B. abortus* clusters included the 96.7% of all *B. abortus* isolates. Clusters were clearly geographically separated, and this highlighted the known epidemiological links among them. *Brucella melitensis* genotypes resulted more heterogeneous; the 3 more representative genetic clusters included 79.7% of all *B. melitensis* isolates. A clear geographical clusterization of genotypes is recognizable only for 1 cluster, whereas the others are more widespread across Southern Italy. The genetic characterization of *Brucella* strains isolated from animals may be a useful tool to better understand the epidemiology and dissemination patterns of this pathogen through host populations.

Caratterizzazione genetica dei cluster geografici di Brucella melitensis *e* Brucella abortus *in Italia*

Parole chiave
MLVA,
Brucella abortus,
Brucella melitensis,
Genotipizzazione,
Italia,
Ruminanti.

Riassunto
La diversità genetica di ceppi di *Brucella melitensis* e *Brucella abortus* isolati in 199 bovini e ovini da 156 focolai di brucellosi verificatisi in 8 regioni del Sud Italia nel 2011 è stata determinata utilizzando una metodologia MLVA-16. L'esistenza di possibili cluster genetici è stata verificata attraverso una analisi gerarchica di cluster basata su un '*single link*', la quale è strettamente legata al '*minimum spanning tree*'. L'analisi è stata condotta utilizzando la matrice di distanza ponderata di Hamming. Tutti i calcoli sono stati effettuati utilizzando il software R e le librerie aggiuntive phangorn e Cluster. Per un numero di cluster che va da 2 a 15 è stata calcolata la larghezza media di silhouette. Il numero di cluster adottato è stato identificato in base alla larghezza media massima della silhouette. Per *B. abortus* e *B. melitensis* sono stati rispettivamente identificati 6 e 11 gruppi genetici. Tre cluster di *B. abortus* su sei comprendono il 96,7% di tutti gli isolati di *B. abortus* e sono chiaramente separati dal punto di vista geografico, mettendo in evidenza note correlazioni epidemiologiche fra loro. I genotipi di *B. melitensis* sono risultati più eterogenei tra loro e i tre cluster genetici più rappresentativi comprendono il 79,7% di tutti gli isolati di *B. melitensis*. Una chiara clusterizzazione geografica dei genotipi è riconoscibile solo per un cluster, mentre gli altri sono più ampiamente diffusi nell'ambito dell'Italia meridionale. La caratterizzazione genetica di ceppi *Brucella* isolati da animali può essere un utile strumento per meglio comprendere l'epidemiologia e i modelli di diffusione di questo patogeno attraverso le popolazioni animali ospiti.

Introduction

The *Brucella* genus comprises Gram-negative bacteria responsible of brucellosis, a worldwide zoonosis disease resulting in significant economic losses due to abortion and infertility in livestock (cattle, goats, and sheep) (Corbel 1997, Pappas *et al.* 2006). The disease can be transmitted to humans directly by contact with infected animals or indirectly by contaminated dairy products. Brucellosis occurs as a sub-acute or chronic illness, which is generally not lethal in previously healthy patients, and can result in a wide variety of manifestations and significant morbidity if the diagnosis is unobserved and treatment is not rapidly started (Pappas *et al.* 2006). Ten species are recognized within the genus *Brucella* (Euzeby 2010), *Brucella abortus* (7 biovars), which preferentially infects cattle; *Brucella melitensis* (3 biovars) infecting sheep and goats; *Brucella suis* (5 biovars) preferentially infecting pigs; *Brucella canis*, which affects dogs; *Brucella ovis*, which affects sheep and goats; *Brucella neotomae*, which infects the desert wood rat; *Brucella microti* affecting the common vole (Scholz *et al.* 2008); *Brucella ceti*, which concerns cetaceans; *Brucella pinnipedialis*, which infects seals (Foster *et al.* 2007); finally *Brucella inopinata*, which was isolated from a human breast implant infection (Scholz *et al.* 2010). Beside this, a recent species, *Brucella papionis*, isolated from baboons, has also been described (Whatmore *et al.* 2014). Each species has distinctive host preferences, pathogenicity, and epidemiology.

As brucellosis control in animal reservoirs is the key to its control in humans, the knowledge of the prevailing genotypes of *Brucella* spp. in a country is an important epidemiological tool for the formulation of policies and strategies (Di Giannatale *et al.* 2006). Current characterization of *Brucella* at species and biovar levels can be performed by differential tests based on phenotypic characterization of lipopolysaccharide (LPS) antigens, phage typing, dye sensitivity, requirement for CO_2, H_2S production, and metabolic properties (Alton *et al.* 1988). Molecular tests with low-resolution typing methods allow for assigning species without definition of biovars (Bricker *et al.* 1994, Hinic *et al.* 2008).

However, these methods have limited value for epidemiological trace-back investigations due to the genetically monomorphic nature of *Brucella* species (Gandara *et al.* 2001). The analysis of the published genome sequences of *B. melitensis* 16M, *B. suis* 1330, and *B. abortus* 9-941 (Whatmore *et al.* 2006, Whatmore *et al.* 2007) resulted in the identification of polymorphic loci. This allowed for the development of schemes based on multilocus sequence typing (MLST) (Bricker *et al.* 2003) and multiple-locus VNTR analysis (MLVA) (Whatmore *et al.* 2006, Le Flèche *et al.* 2006, Al Dahouk *et al.* 2007).

The aims of this study were to characterize molecularly animal isolates of *B. abortus* and *B. melitensis* isolated in 2011 from domestic ruminants in Italy; to evaluate possible correlations between different genotypes as identified with the MLVA-16 technique and different geographical patterns of distribution for these zoonotic agents, and to provide a supplementary epidemiological tool for disease control and eradication.

Materials and methods

Brucella isolates

A total of 199 *Brucella* isolates from animal brucellosis cases identified in Italy during 2011 were included in the study. Strains were isolated from 156 outbreaks which occurred in farms placed in the following regions of Southern Italy: Sicily, Calabria, Apulia, Basilicata, Molise, Campania, and Abruzzo.

One hundred and twenty *B. abortus* isolates were collected from 91 farms and were isolated from 105 cattle, 14 Mediterranean Italian water buffalo (*Bubalus bubalis*) and 1 sheep, respectively.

Seventy-nine *B. melitensis* isolates were collected from 65 farms and were isolated from 56 sheep, 15 goats, and 8 cattle, respectively.

All strains were isolated in the local Istituti Zooprofilattici Sperimentali and sent to the Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise 'G. Caporale', Teramo, Italy, National Reference Centre for Brucellosis for typing. Strains were grown onto serum dextrose agar and examined microscopically using the Gram stain, biochemical (catalase, oxidase and urease activity) and motility tests, according to international standards (OIE 2009). Species and biovar identification were performed by evaluating agglutination with *Brucella* anti-A, anti-M, and anti-R monospecific antisera (Veterinary Laboratories Agency, Weybridge, United Kingdom), by evaluating the production of H_2S, the CO_2 dependence, as well as by evaluating the growth in the presence of thionine and basic fuchsin (OIE 2009).

Brucella DNA was extracted using Chelex-based Istagene Matrix (Bio-Rad Laboratories Inc., Hercules, California, USA). Results were confirmed with *abortus-melitensis-ovis-suis* (AMOS) multiplex polymerase chain reaction (PCR) (Bricker and Halling 1994, Bricker and Halling 1995) and polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) techniques (Cloeckaert *et al.* 1995, Vizcaino *et al.* 1997). Biovar assignation was obtained by RFLP of *omp2a* and *omp2b* analyses (Cloeckaert *et al.* 1995). For RFLP, the *omp2a*

and *omp31* PCR products were digested using *NcoI* and *AvaII* restriction enzymes, respectively (Promega Corporation, Madison, Wisconsin, USA) (Di Giannatale *et al.* 2006).

MLVA genotyping

Samples were genotyped using the MLVA-16 panel of Le Flèche and colleagues (Le Flèche *et al.* 2006), with modifications by Al Dahouk and colleagues (Al Dahouk *et al.* 2007). Loci considered were BRUCE_06, BRUCE_08, BRUCE_11, BRUCE_12, BRUCE_42, BRUCE_43, BRUCE_45, BRUCE_55, BRUCE_18, BRUCE_19, BRUCE_21, BRUCE_04, BRUCE_07, BRUCE_09, BRUCE_16, BRUCE_30.

Amplification of MLVA-16 loci was performed using multiplex PCRs as described by Garofolo and colleagues (Garofolo *et al.* 2013a). PCR amplifications were performed in a total volume of 10 µl containing 1.50 ng DNA, 1× Type-it microsatellite PCR Master Mix (Qiagen Srl, Milan, Italy), and proper concentration of each fluorescent primer pairs according to Garofolo and colleagues (Garofolo *et al.* 2013a). Thermal cycling, conducted on a GeneAmp 9700 thermal cycler (Applied Biosystems, Carlsbad, California, USA), was performed as follows: initial heating at 95 °C for 5 minutes, 30 cycles denaturation at 95 °C for 30 seconds, annealing at 60 °C for 90 seconds and extension at 72 °C for 30 seconds. A final extension step at 60 °C for 45 minutes and 20 °C for 120 minutes was run to reduce artefacts such as stutter and non-templated 3' A nucleotide additions. Fragments were then separated through capillary electrophoresis on an ABI 3500 instrument with POP 7 polymer. Data analysis was done using Genemapper 4.1 (Applied Biosystems, Carlsbad, California, USA) to assign for each VNTRs the actual allele.

Statistical analysis

In order to validate the robustness of MLVA clusters, the 16 MLVA variables were processed through a hierarchical cluster analysis using weighted dissimilarity. The Hamming weighted distance matrix was adopted in the analysis, given that single versus multiple repeat mutation ratios and insertion versus deletion mutation ratios were similar. Moreover, in a local investigation, it is expected that strains considered in the analysis would belong to a common ancestor (likely an Italian ancestor) and, in this context, the use of different mathematical distances, such as the Euclidean distance, is not expected to change the figure. The 16 MLVA variables were weighted as follows: loci BRUCE_06, BRUCE_08, BRUCE_11, BRUCE_12, BRUCE_42, BRUCE_43, BRUCE_45 and BRUCE_55 a weight of 10 was assigned; for loci BRUCE_18, BRUCE_19, and BRUCE_21 a weight of 5 was assigned; for loci BRUCE_04, BRUCE_07, BRUCE_09, BRUCE_16, and BRUCE_30 a weight of 1 was assigned. Weights were attributed according to literature (Al Dahouk *et al.* 2007). However, the proportion of values was changed to avoid problems in R program by using decimals for cluster analysis. The values 1, 5, and 10 were used, which are proportional to 0.2, 1, and 2 as described by Al Dahouk and colleagues (Al Dahouk *et al.* 2007). Using the 'bottom-up' approach, each object has been initially identified as a single cluster (singleton), then the algorithm has proceeded iteratively, joining at each stage the 2 most similar clusters, and continuing until a final single cluster containing all objects was created. The clustering method used was based on 'Single link' (*i.e.* the distance chosen between objects has been the smaller among the distances calculated between all the elements of the cluster), which is closely related to the minimum spanning tree and adopts a 'friends of friends' clustering strategy. All calculations were performed using R[1] and the additional libraries phangorn (Schliep 2011) and Cluster (Maechler *et al.* 2012). For a number of clusters ranging from 2 to 15 the average silhouette width was calculated in order to identify the most appropriate number of clusters derivable from the analysis, as well as their validity. The appropriate number of clusters was identified with the 1 resulting in the maximum average silhouette width (s_i) value (Rousseeuw 1987). The overall average silhouette value (0.5) is a value at the limit of acceptability for the validity of this clustering.

Results

Brucella species and biovar isolated

Brucella melitensis biovar 3, *B. abortus* biovar 1, biovar 3, and RB51 vaccine strain were identified in cattle (113 strains, 56.8%), water buffalo (14 strains, 7.0%), sheep (57 strains, 28.6%), and goats (15 strains, 7.5%). Results are detailed in Table I.

Cluster analysis for *B. abortus*

The dendrogram resulting from the hierarchical cluster analysis for the 120 *B. abortus* isolates is shown in Figure 1.

The results of the average silhouette width calculation for a possible number of *B. abortus* clusters ranging

[1] R Development Core Team, 2007. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org.

Table I. *Phenotypical identification of* Brucella *strains isolated in Southern Italy in 2011.*

Brucella species and biovar	Host species				Total
	Cattle	Water buffalo	Sheep	Goat	
B. abortus biovar 1	3	3	-	-	**6 (3.0%)**
B. abortus biovar 3	102	10	1	-	**113 (56.8%)**
B. abortus strain RB51	-	1	-	-	**1 (0.5%)**
B. melitensis biovar 3	8	-	56	15	**79 (39.7%)**
Total	**113 (56.8%)**	**14 (7.0%)**	**57 (28.6%)**	**15 (7.5%)**	**199**

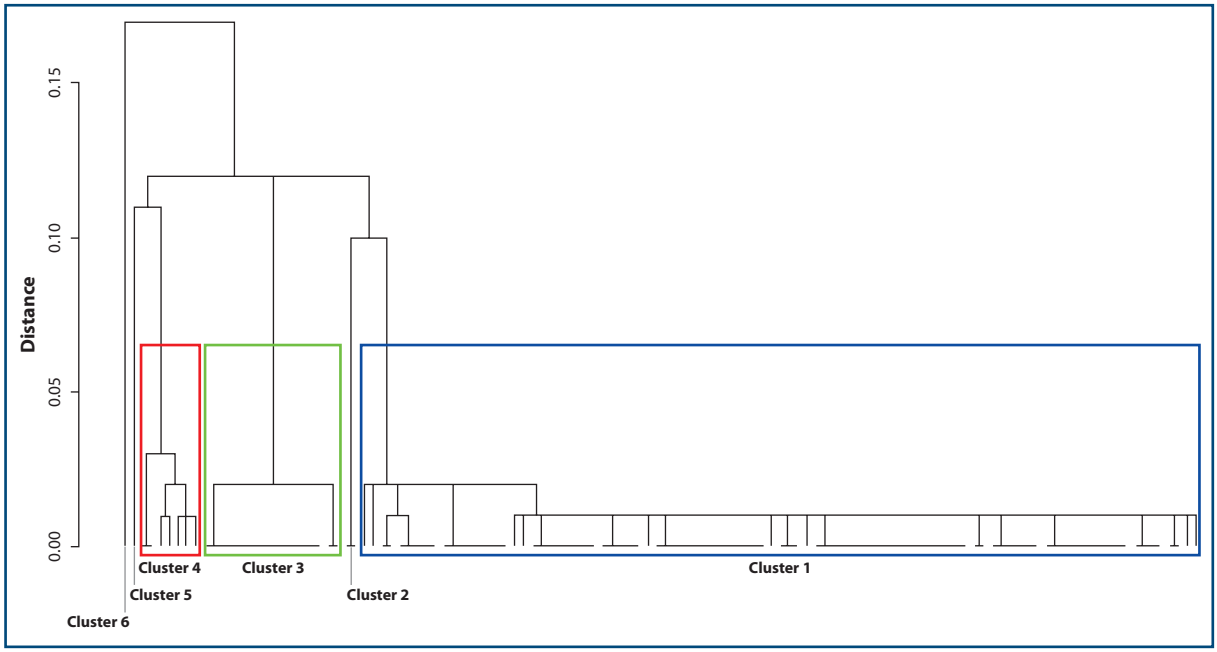

Figure 1. *Dendrogram resulting from the hierarchical cluster analysis of 120* B. abortus *isolates in Southern Italy in 2011 using the Hamming weighted distance matrix.*

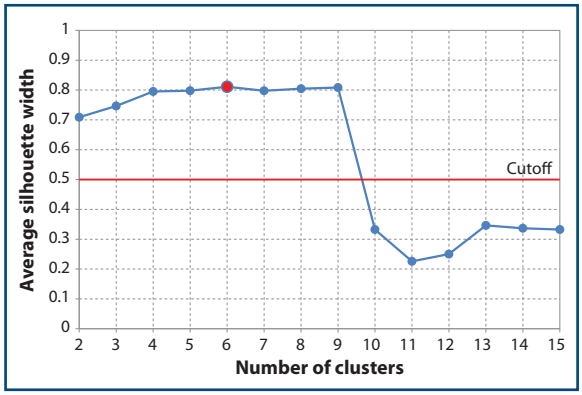

Figure 2. *Results of the average silhouette width calculation for a possible number of* B. abortus *clusters ranging from 2 to 15.*

from 2 to 15 are shown in Figure 2. For *B. abortus* the best value of average silhouette width (0.81) is identified with a subdivision in 6 clusters.

The silhouette plot for the 6 *B. abortus* clusters is shown in Figure 3. Clusters containing just 1 element are by definition set to 0. The cluster identified as cluster 1 includes the majority of the isolates considered (95 out of 120; 77.9%) suggesting the low genetic variability of *B. abortus* strains isolated in Italy in 2011. Classical typing methods identified all the strains of cluster 1 as *B. abortus* biovar 3. Cluster 2 is grouping 2 *B. abortus* biovar 3 strains both isolated from water buffalo. Cluster 4 is grouping 7 strains identified as *B. abortus* biovar 1. Figure 3 includes also the average silhouette width for each cluster. Values of average silhouette width above 0.71 are considered as indicative of a good validity of the clustering. The results show that this value is reached by the first 4 clusters (clusters 1-4; Figure 3). The 2 remaining clusters are composed each one by 1 strain which is completely different from any other strain considered. In particular, cluster 5 was a *B. abortus* RB51 strain isolated from a water buffalo.

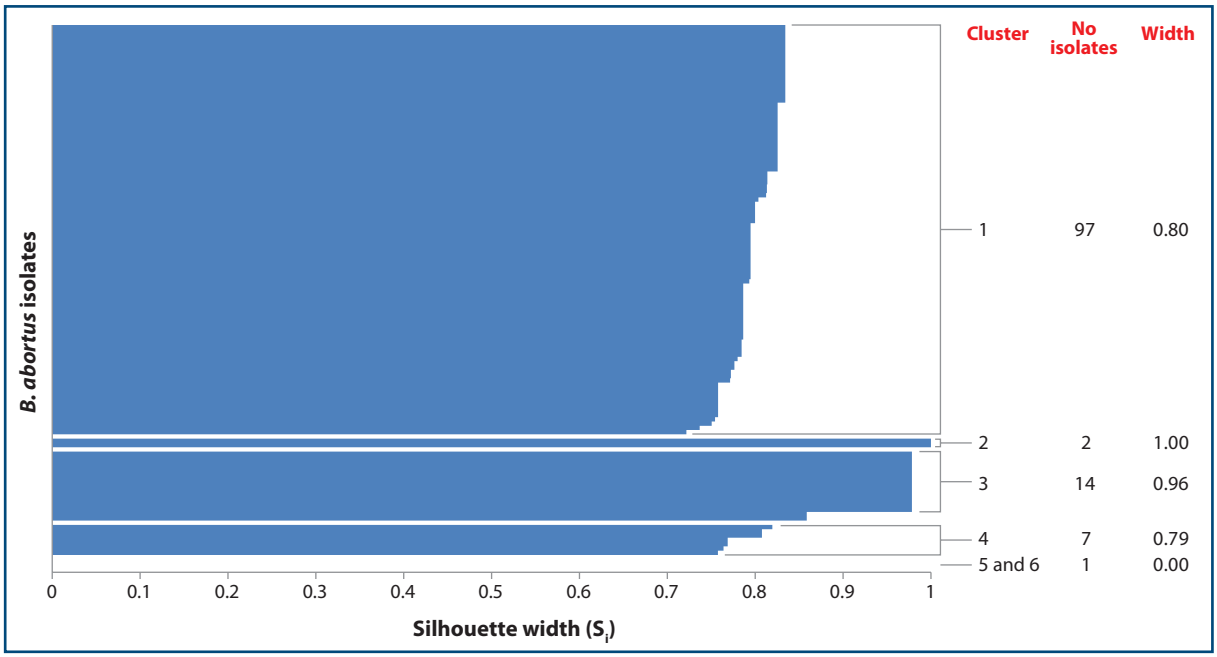

Figure 3. *Silhouette plot for 6* B. abortus *clusters including the average silhouette width for each cluster.* The overall average silhouette width is 0.81.

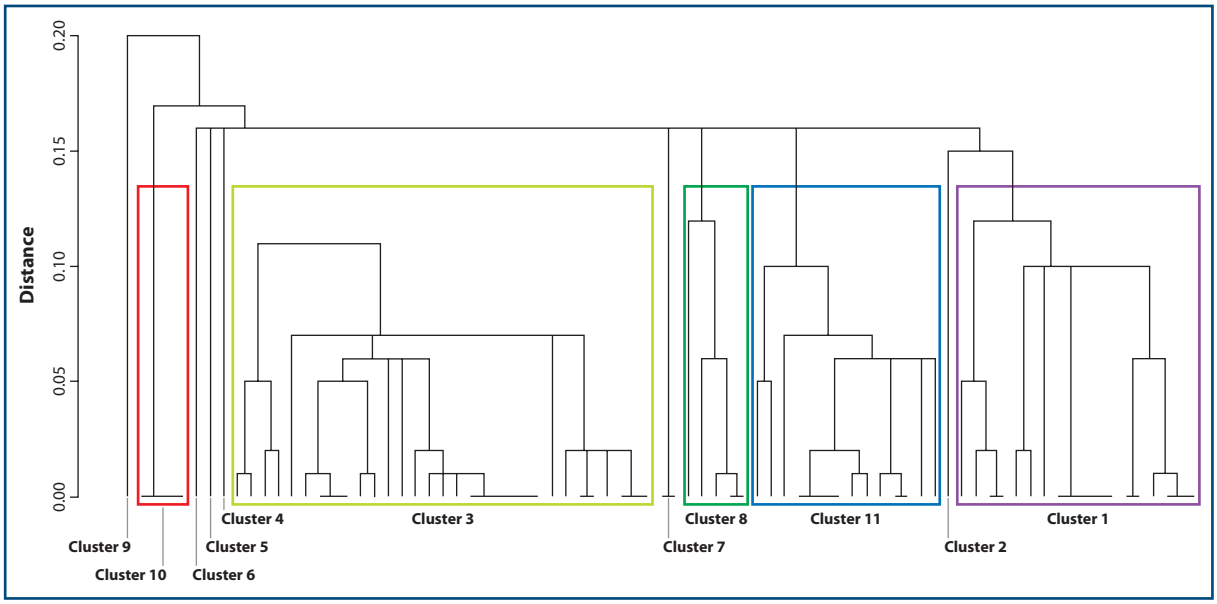

Figure 4. *Dendrogram resulting from the hierarchical cluster analysis of 79* B. melitensis *isolates using the Hamming weighted distance matrix.*

Cluster analysis for *B. melitensis*

The dendrogram resulting from the hierarchical cluster analysis for the 79 *B. melitensis* isolates is shown in Figure 4.

The results of the average silhouette width calculation for a possible number of *B. melitensis* clusters ranging from 2 to 15 are shown in Figure 5. For *B. melitensis*, the best value of average silhouette width (0.5) is identified with a subdivision in 11 clusters.

The silhouette plot for the 11 *B. melitensis* clusters is shown in Figure 6. Cluster 3 includes the higher number of isolates (31 out of 79; 39.2%). However, the high number of clusters identified and the consequent low number of isolates in most clusters suggest a high genetic variability of *B. melitensis* strain isolated in Southern Italy in 2011. The figure includes also the average silhouette width for each cluster. Values of average silhouette width above 0.51 are considered as indicative of an acceptable validity of the clustering. Results show that this value is reached by 4 clusters (7, 8, 10, and 14; Figure 6). The 7 remaining clusters have an average silhouette value between 0.26 and 0.5, which is indicative of a weak validity of grouping.

Figure 5. *Results of the average silhouette width calculation for a possible number of* B. melitensis *clusters ranging from 2 to 15.*

Geographical distribution of *B. abortus* clusters in Southern Italy

For the geographical representation of the data, the first 3 clusters with the higher number of *B. abortus* isolates have been chosen (*i.e.* clusters 1, 3 and 4 with 97, 14, and 7 isolates respectively). These clusters included 96.7% of all *B. abortus* isolates considered. The geographical location of the farm of origin of the isolates is shown in Figure 7. While isolates from cluster 1 are spread all over Southern Italian territories, clusters 3 and 4 seem to have a distribution confined in a limited area.

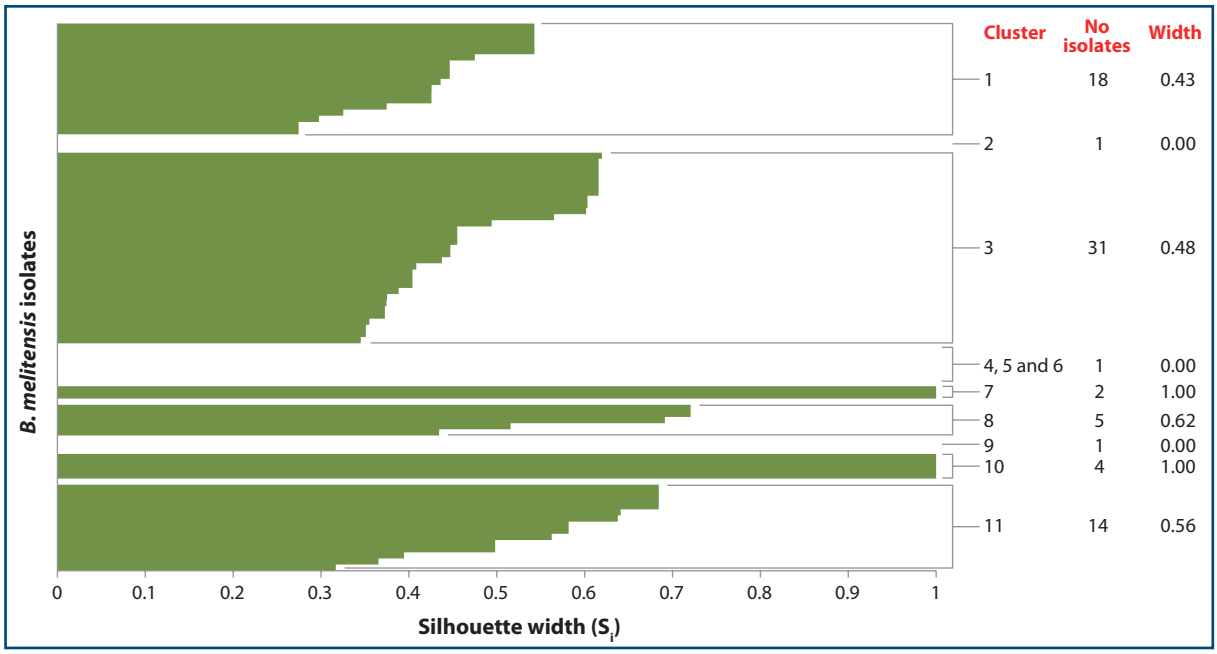

Figure 6. *Silhouette plot for 11* B. melitensis *clusters including the average silhouette width for each cluster.* The overall average silhouette width is 0.5.

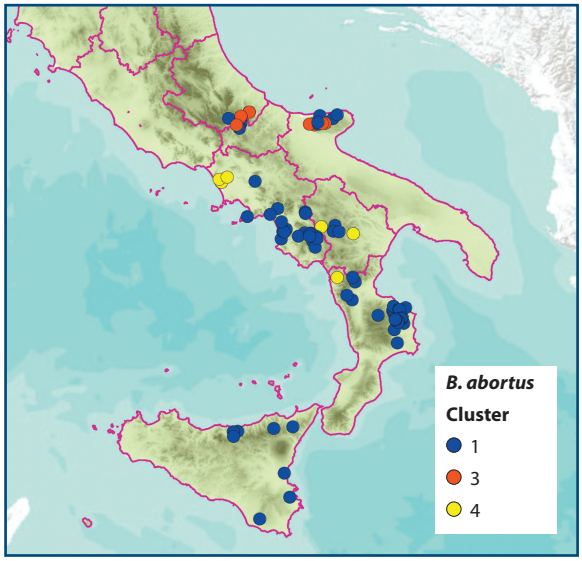

Figure 7. *Geographical distribution of* B. abortus *isolated in 2011 in Italy according to clusters 1, 3, and 4.*

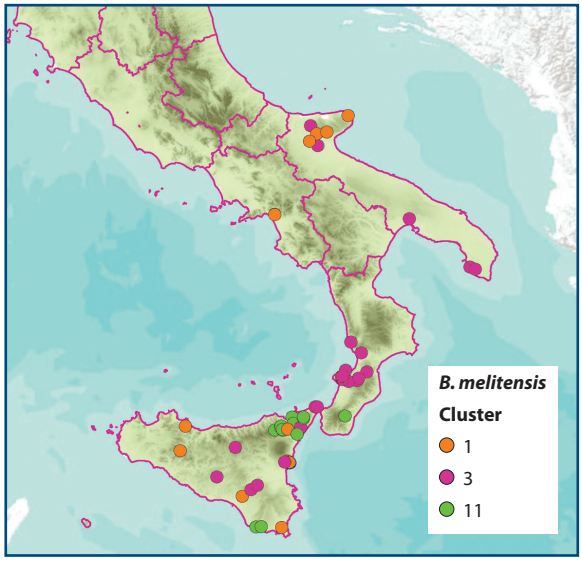

Figure 8. *Geographical distribution of* B. melitensis *isolated in 2011 in Southern Italy according to clusters 1, 3, and 11.*

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Geographical distribution of *B. melitensis* clusters in Italy

For the geographical representation of the data, the first 3 clusters with the higher number of *B. melitensis* isolates have been chosen (*i.e.* clusters 1, 3, and 11 with 18, 31, and 14 isolates respectively), regardless to their average silhouette value. These clusters included 79.7% of all *B. melitensis* isolates considered. The geographical location of the farm of origin of the isolates is shown in Figure 8. While isolates from clusters 1 and 3 are spread all over Southern Italian territories, clusters 11 seem to have a distribution confined in a limited area.

Discussion

Brucellosis remains an important zoonosis in Italy with a considerable number of human cases reported every year, mainly due to *B. melitensis*. Between 1998-2010, 8,483 cases of human brucellosis have been notified in Italy, the 89% of them occurring in Southern Italian regions (Apulia, Campania, Calabria, and Sicily). The incidence of human cases of brucellosis, in the period 2007-2010, seems to be stable around 0.3 cases/100,000 inhabitants (Graziani *et al.* 2013). In this study we have characterized 199 *Brucella* strains isolated from animals (cattle, water buffalo, sheep, and goats) in the context of 156 outbreaks which occurred in farms placed in Southern Italian regions in 2011.

The analysis performed using a strict statistical computation, broadly confirms the previous results from Garofolo and colleagues (Garofolo *et al.* 2013b). To assess phylogeny, the MLVA-11 genotype is normally used, and the cluster analysis here performed revealed only slight differences. Indeed for *B. abortus* and *B. melitensis*, 6 and 11 genetic clusters were identified, in contrast with 7 and 15 MLVA-11 genotypes respectively. Three out of 6 *B. abortus* clusters include 96.7% of all *B. abortus* isolates and they are geographically separated, suggesting the existence of epidemiological links among them. In particular cluster 3 (visualized as red dots in Figure 7) is epidemiologically linked to cattle movements to pastures in Summer, which favour *Brucella* transmission. More investigations are ongoing for better explaining other *B. abortus* clusterization observed in Southern Italy.

Genotypes of *B. melitensis* resulted more heterogeneous. The 3 more representative genetic clusters include 79.7% of all *B. melitensis* isolates. A geographical clusterization of genotypes is recognizable only for 1 cluster, whereas the others are more widespread across Southern Italy.

The phylogeography of *Brucella* in Europe, especially in Italy, has been challenging to elucidate, likely due to widespread movement of infected animals across regional, national, and international borders (Garofolo *et al.* 2013b). The different clones shown for *B. melitensis* are likely a result of the frequent introduction and reintroduction of this pathogen across Italy. The trade of animals and their products were easier in the past decades posing few restrictions for *B. melitensis*. Probably the relatively small size of goats and sheep and their low cost allowed for a widespread movement of these animals and their pathogens. In contrast, *B. abortus* had a clonal population with much less diversity, possibly due either to fewer and more geographically limited introductions into Italy or to lower discrimination power of MLVA-16 panel for *B. abortus*.

The epidemiological analysis of genetic clusters may help to better understand the pathogen transmission patterns between animal sub-populations, giving precious indication for the prevention of infection spread. Further studies are needed, including the analysis of a larger temporal window, to better clarify the spatial distribution of *Brucella* genotypes observed in our results. There is also the need to standardize a proper metadata analysis to better understand MLVA cluster dispersion; the statistical approach here proposed is a first step further. The average silhouette width helped us to define clusters using a system less prone to interpretations. The deployment of this approach within large studies focusing on thousands of strains could help to defining clusters and even detect misidentified strains when they fall in inconsistent clusters. These methodologies will help us in managing data in a puzzling scenario for lasting the eradication of brucellosis in Southern Italy.

Given that we have now entered a new genomic era of next generation sequencing, comparative whole genome sequence analysis of potential novel *Brucella* species and *Brucella*-like organisms will provide a better understanding of the evolution, host specificity and pathogenicity of this medically important genus (Scholtz and Vergnaud 2013).

The genetic characterization of *Brucella* strains isolated from animals may be a useful tool for better understanding the epidemiology and dissemination patterns of this pathogen through host populations.

Although MLVA-16 has been recognised as a useful tool for outbreak investigation, in the foreseeable future merging these data with whole genome sequencing will elucidate the molecular epidemiology of these globally important pathogens to better define and resolve the significant clusters. Nevertheless further studies on *Brucellae* isolates from Italy are needed to understand the molecular evolution within the country and to discover the global relationship.

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References

Al Dahouk S., Le Flèche P., Nockler K., Jacques I., Grayon M., Scholz H.C., Tomaso H., Vergnaud G. & Neubauer H. 2007. Evaluation of *Brucella* MLVA typing for human Brucellosis. *J Microbiol Methods*, **69**, 137-145.

Bricker B.J. & Halling, S.M. 1994. Differentiation of *Brucella abortus* bv. 1, 2, and 4, *Brucella melitensis, Brucella ovis* & *Brucella suis* bv. 1 by PCR. *J Clin Microbiol*, **32**, 2660-2666.

Bricker B.J., Ewalt D.R. & Halling S.M. 2003. *Brucella* 'Hoof-Prints': typing by multi-locus analysis of variable number tandem repeats (VNTRs). *BMC Microbiol*, **3**, 15.

Bricker B.J. & Halling S.M. 1994. Differentiation of *Brucella abortus* bv. 1, 2, and 4, *Brucella melitensis, Brucella ovis*, and *Brucella suis* bv. 1 by PCR. *J Clin Microbiol*, **32**, 2660-2666.

Bricker B.J. & Halling S.M. 1995. Enhancement of the *Brucella* AMOS PCR assay for differentiation of *Brucella abortus* vaccine strains S19 and RB51. *J Clin Microbiol*, **33**, 1640-1642.

Cloeckaert A., Verger J.M., Grayon M. & Grèpinet O. 1995. Restriction site polymorphism of the genes encoding the major 25 Kda and 36 Kda outer membrane proteins of *Brucella*. *Microbiol*, **141**, 2111-2121.

Corbel M.J. 1997. Brucellosis: an overview. *Emerg Infect Dis*, **3**, 213-221.

Di Giannatale E., De Massis F., Ancora M., Zilli K. & Alessiani A. 2006. Typing of *Brucella* field strains isolated form livestock populations in Italy between 2001 and 2006. *Vet Ital*, **44**, 383-388.

Euzeby J.P. 2010. List of prokaryotic names with standing in nomenclature - genus *Brucella*. http://www.bacterio.cict.fr/b/brucella.html.

Foster G., Osterman B.S., Godfroid J., Jacques I. & Cloeckaert A. 2007. *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*, **57**, 2688-2693.

Gandara B., Merino A.L., Rogel M.A. & Martinez-Romero E. 2001. Limited genetic diversity of *Brucella* spp. *J Clin Microbiol*, **39**, 235-240.

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

Garofolo G., Di Giannatale E., De Massis F., Zilli K., Ancora M., Cammà C., Calistri P. & Foster J.T. 2013b Investigating genetic diversity of *Brucella abortus* and *Brucella melitensis* in Italy with MLVA-16. *Infect Genet Evol*, **19**, 59-70.

Graziani C., Mancini F.R., Adone R., Marianelli C., Pasquali P., Rizzo C., Bella A., De Massis F., Danzetta M.L., Calistri P., Primavera A., Ruocco L. & Busani L. 2013. La brucellosi in Italia dal 1998 al 2011. Roma: Istituto Superiore di Sanità (Rapporti ISTISAN 13/45), 75 pp.

Hinic V., Brodard I., Thomann A., Cvetnić Z., Makaya P.V., Frey J. & Abril C. 2008. Novel identification and differentiation of *Brucella melitensis, B. abortus, B. suis, B. ovis, B. canis,* and *B. neotomae* suitable for both conventional and real-time PCR systems. *J Microbiol Methods*, **75**, 375-378.

Le Flèche P., Jacques I., Grayon M., Al Dahouk S., Bouchon P., Denoeud F., Nöckler 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.

Maechler M., Rousseeuw P., Struyf A., Hubert M. & Hornik K. 2012. Cluster: cluster analysis basics and extensions. R package version 1.14.2.

Maquart M., Le Flèche P., Foster G., Tryland M., Ramisse F., Djønne B., Al Dahouk S., Jacques I., Neubauer H., Walravens K., Godfroid J., Cloeckaert A. & Vergnaud G. 2009. 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*, **9**, 145.

Pappas G., Papadimitriou P., Akritidis N., Christou L. & Tsianos E.V. 2006. The new global map of human Brucellosis. *Lancet Infect Dis*, **6**, 91-99.

Rousseeuw P.J. 1987. Silhouettes: a graphical aid to the interpretation and validation of cluster analysis. *J Comput Appl Math*, **20**, 53-65.

Schliep K.P. 2011. Phangorn: phylogenetic analysis in R. *Bioinformatics*, **27**, 592-593.

Scholz H.C. & Vergnaud G. 2013. Molecular characterisation of *Brucella* species. *Rev Sci Tech Off Int Epiz,* **32**, 149-162.

Scholz H.C., Hubalek Z., Sedláček I., Vergnaud G., Tomaso H., Al Dahouk S., Melzer F., Kämpfer P., Neubauer H., Cloeckaert A., Maquart M., Zygmunt M., Whatmore A.M., Falsen E., Bahn P., Göllner C., Pfeffer M., Huber B., Busse H.-J. & Nöckler K. 2008. *Brucella microti* sp. nov., isolated from the common vole *Microtus arvalis*. *Int J Syst Evol Microbiol*, **58**, 375-382.

Scholz H.C., Nöckler K., Göllner C., Bahn P., Vergnaud G., Tomaso H., Al Dahouk S., Kämpfer P., Cloeckaert A., Maquart M., Zygmunt M., Whatmore A.M., Pfeffer M., Huber B., Busse H.-J. & Kumar De B. 2010. *Brucella inopinata* sp. nov., isolated from a breast implant infection. *Int J Syst Evol Microbiol*, **60**, 801-808.

Vizcaino N., Verger J.M., Grayon M., Zygmunt M.S. & Cloeckaert A. 1997. DNA polymorphism at the omp-31

locus of *Brucella* spp.: evidence for a large deletion in *Brucella abortus*, and other species-specific markers. *Microbiol*, **143**, 2913-2921.

Whatmore A.M., Davison N., Cloeckaert A., Al Dahouk S., Zygmunt M.S., Brew S.D., Perrett L.L., Koylass M.S., Vergnaud G., Quance C., Scholz H.C., Dick E.J., Hubbard G. & Schlabritz-Loutsevitch N.E. 2014. Full taxonomic description of the proposed eleventh *Brucella* species, *Brucella papionis* sp. nov., isolated from baboons. Proceedings of the Brucellosis 2014 International Research Conference, Berlin, 9-12 September 2014. 21.

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

Whatmore A.M., Shankster S.J., Perrett L.L., Murphy T.J., Brew S.D., Thirlwall R.E., Cutler S.J. & MacMillan A.P. 2006. Identification and characterization of variable-number tandem-repeat markers for typing of *Brucella* spp. *J Clin Microbiol*, **44**, 1982-1993.

World Organisation for Animal Health (OIE). 2009. Manual of diagnostic tests and vaccines for terrestrial animals. Chapter 2.4.3. Bovine brucellosis. OIE Paris. http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.04.03_BOVINE_BRUCELL.pdf.