**Brucella: Molecular Diagnostic Techniques in Response to Bioterrorism Threat**

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**Abstract**

Brucellosis, a worldwide zoonosis caused by members of the genus *Brucella*, is responsible of a considerable human morbidity and economic losses. Although the disease is associated with low mortality and has a relative limited medical impact, *Brucella* spp., particularly *B. melitensis* and *B. abortus*, have been also reported as possible biological weapons. A prompt detection and identification of involved biological agents and the following discrimination between natural outbreaks and/or intentional release of micro-organism, represents the crucial point for an effective response. Furthermore, being members of the genus *Brucella* genetically homogeneous, the development of accurate strain typing methods is essential in order to investigate the source of an epidemic event. The aim of this paper is to provide an overview of the current molecular diagnostic tools developed as response to bioterrorism episodes.

**Key words:** Bioterrorism; *Brucella*; Molecular genotyping;

**Introduction**

*Brucella* is the causative agent of brucellosis (also named Malta fever or undulant fever), a zoonotic infection. The genus *Brucella* belongs to the order Rhizobiales within the class alpha-proteobacteria [1]. Members of the genus *Brucella* are Gram negative, aerobic, non-spore-forming, non-motile coccobacilli whose preferred niche is intracellular. Six species and biovars were classically differentiated on the basis of distinct host specificity and differential tests based on phenotypic characterization of lipopolysaccharide (LPS) antigens, phase typing, dye sensitivity, requirement for CO2, H2S production and metabolic proper ties. *B. melitensis*, with three biovars currently recognised, *B. abortus*, with eight biovars, and *B. suis*, with five biovars, causative agents of bovine, small ruminant and swine brucellosis respectively, are recognised as the most economically significant pathogens of the group and generally considered pathogenic for humans in order of virulence [2,3]. The other three species are *B. canis* and *B. ovis*, associated respectively with canine brucellosis and ruminant epididymitis and *B. neotomae*, associated with the desert wood rat [2]. Recently, new species of *Brucella* have been described: *B. ceti* and *B. pinnipedialis* isolated from marine mammals [4-6], *B. microti*, isolated from voles [7], foxes and soil [8,9], and finally *B. inopinata*, isolated from a human breast implant infection [10,11]. An unusual gram-negative, non-motile *Brucella*-like coccoid bacillus (BO2) isolated from a lung biopsy, showed unique similarity with the *B. inopinata* strain [12].

**Epidemiology**

Brucellosis occurs worldwide in both human and animals. Distribution of human brucellosis has changed over the last fifty years because of different factors as sanitary, socioeconomic, and political conditions, together with the increase of international travel and population migration [13]. Several areas traditionally considered endemic e.g. France and Israel, have achieved the control of the disease while new foci of human brucellosis have emerged, particularly in central Asia and Middle East (eg, Syria) [14]. Nowadays the infection is most common in the Mediterranean basin, the Middle East, India, Central Asia, Mexico, and Central and South America [15] while in Northern Europe, Australia, the USA and Canada control programs allowed eradicating the infection [2]. In Europe the distribution of brucellosis varies widely. Brucellosis-free status has been granted by the European Union (EU) to Sweden, Denmark, Finland, Germany, UK (excluding Northern Ireland), Austria, Netherlands, Belgium, and Luxembourg. Norway and Switzerland are also considered brucellosis-free countries [16], while the Mediterranean basin is known to be an endemic region of human brucellosis [17]. Indeed, in Italy human brucellosis has travelled to the south, because of socioeconomic factors [18], as Portugal and Greece, endemic areas, characteristically the poorest regions of the EU [19]. In the Northern United States, brucellosis cases are due mainly to disease imported through international travel or infected food preparations coming from endemic areas. In the rest of the United States (USA) *B. melitensis* is the main responsible of *Brucella* infections, especially in the Hispanic population, localised in areas neighbouring Mexico [14]. Indeed, since Mexico is the principal reservoir of infection, the immigration into the USA prevents the eradication of the disease. In Africa the brucellosis is endemic, especially in north Africa, where sanitary data are available, while in the most African countries the fragmentary collection of clinical data doesn’t allow to have reliable status of the prevalence [14].

**Brucellosis and bioterrorism**

*Brucella* species, particularly *B. melitensis* and *B. suis*, have traditionally been considered biological weapons, although brucellosis is characterized by a long incubation period, often asymptomatic infections and low mortality. Indeed, the airborne transmission through mucous membranes such as the conjunctiva, oropharynx, respiratory tract or the transmission through skin abrasions make *Brucella* highly contagious, as demonstrated by efficient human airborne transmission during abortions of infected animals or aerosolisation in laboratory manipulations [20,21]. Furthermore, factors as the low number of...
bacteria constituting an infectious aerosol dose (10–100 organism), the nonspecific clinical symptoms of brucellosis, the worldwide circulation of the infection, the onset of chronic debilitating disease, make of *Brucella* spp. a category B bioterroristic agent, according the Center for Disease Control and Prevention definition [22]. Although *Brucella* is sensitive to inactivation standard methods as heating and disinfectants, it often survives for up to two years in the environment [20].

The most virulent of several strains of the *Brucella* bacteria, code name US, was the most advanced and the only standardized agent fill by the end of the 1950. By the summer of 1951 the Chemical Corps Biological Department scheduled the production of *B. suis* and *B. melitensis*. In 1954, *B. suis* became the first agent weaponised by the USA and tested on animals. By 1955, the USA filled cluster bombs with this agent for the US Air Force at the Pine Bluff Arsenal in Arkansas [23]. In 1967 the development of *Brucella* as a bioweapon was stopped, and Richard Nixon on 1969 in the Statement on Chemical and Biological Defense Policies and Programs unilaterally renounced to use chemical weapons and the banned the development of all biological weapons. A preliminary treaty prohibiting the development, storage acquisition of biological weapons was completed in 1972 and ratified as Biological and Toxin Weapons Convention (BTWC) in 1975 from 144 countries. However several nations of the Middle East did not sign the treaty and Soviet Union, in spite of the Convention, expanded its biological weapons program [24].

In 1999 a case of brucellosis was reported in a 38-year-old woman who resided in New Hampshire. Even if the atypical clinical presentation and suspicious circumstances raised the possibility of a case of bioterrorism, it was impossible for criminal investigators to find evidence of biological terrorism act. [25]. In fact, the correlation of suspicious cases to a possible attack involving a biological agent represents the mainly difficult in the forensic investigations. An effective public health response to a possible biological terrorism crime or terrorism threat include 1) sensitive, specific, and rapid laboratory diagnosis of patients and characterization of biological agents; 2) early detection through improved surveillance; 3) effective communication; and 4) coordinated local, state, and federal response in the investigation of unusual events or unexplained illnesses [25]. The early detection is essential to ensure a prompt response to a biological terrorist event, but also the discrimination between natural outbreaks and/or intentional release of micro-organism agents is of crucial importance in the context of the bioterrorism. Therefore it is very important to have a strain typing epidemiological tool for source trace back in outbreaks. Characterization of *Brucella* at species and biobar level using differential microbiological approaches for phenotyping often may result in complication interpretation where a more accurate identification is necessary [26,27]. Furthermore, these typing methods are time consuming and potentially hazardous for laboratory oper ators, as *Brucella* spp. need BS3L facilities. Thus, genetic characterization using molecular DNA technology has been developed and several molecular techniques for subtyping have been proposed.

Pathogenesis and clinical diagnosis

*Brucella* species are facultative intracellular bacteria able to multiply within human or animal phagocytes cells, survive to intracellular conditions and escape to the host’s immune system [3,28,29]. Epithelial cells, placental trophoblasts, dendritic cells and macrophages are the target cells [30,31]. Transmission of brucellosis to humans is usually the result of direct or indirect contact via ingestion or inhalation, or through conjunctiva or skin abrasions [15,31,32]. The brucellosis is a typical occupational disease affecting especially farmers, veterinarians, abattoir workers and laboratory per sonnel exposed to aerosolisation [33]. Interhuman transmission is rare and only, some anecdotic cases are reported as following blood exposure, primary exposure to infected tissues or after sexual contact [20]. Human brucellosis present in various forms with signs mostly non-specific and similar in patients whatever the route of transmission. The main symptoms are fever or chills, arthralgia, sweating and hepatomagaly and splenomegaly [34].

Diagnosis

The “gold standard” in the diagnosis of brucellosis is bacterial isolation from blood or bone marrow specimens that requires long cultivation per iods (4 to 7 days up to 40 days) and often the blood cultures are unsuccessful [35]. Serological tests, as serum agglutination test (SAT), Rose Bengal test, complement fixation test, and enzyme-linked immunosorbent assay are still frequently used [36]. Since the routine identification and differentiation of brucellosis suspected specimens, based on culture isolation and phenotypic characterization, requires BS3L protocols for the high risk of laboratory-acquired infections [37,31], molecular methods have been explored in order to overcome these difficulties. Furthermore, the Polimerase Chain Reaction (PCR)-based assays have shown a higher sensitivity respect to the standard microbiological assay for the diagnosis of brucellosis. [15].

Molecular methods for *Brucella* spp. genotyping

PCR specie-specific: PCR DNA-based methods such as gene probes and PCR utilize primers derived from different polymorphic regions in the genomes of *Brucella* species. Different PCR methods for the detection of *Brucella* spp. that utilize primers derived from different polymorphic regions in the genomes of *Brucella* species as i.e. (1) a gene encoding a 31-kDa *B. abortus* antigen which is conserved in all *Brucella* species (primers B4/B5) [38], (2), a sequence 16S rRNA of *B. abortus* (primers F4/R2) [39], (3), a gene encoding an outer membrane protein of 26-kDa (omp2) (primers JPF/JPR and primes P1/P2) [40,41], (4) outer membrane proteins (omp2, omp2a and omp31) [42], (5) proteins of the omp25/omp31 family of *Brucella* spp [43], the entire bp26 gene of *B. melitensis* 16M, encoding the BP26 protein (omp 28) (primers 26A/26B) [44] were described [45,46]. However these techniques allow the differentiation of limited number of species. The comparison of PCR sensitivity for *Brucella* DNA detection shows different values for distinct assays, i.e. the limit of sensitivity was 8 fg for B4/B5, 5 pg for F4/R2 and 20 pg for JPF/JPR [45]. Decrease of PCR sensitivity was observed in presence of human genomic DNA for primers F4/R2 and B4/B5, from 8 fg to 800 fg and from 5 pg to 50 pg respectively, while JPF/JPR were not affected. Another comparison evaluating the sensitivity of the PCR primer pairs B4/B5, JPF/JPR, P1/P2 and 26A/26B, applied in about 5000 samples (buffy coat, whole-blood, and serum) was described [47]. The results of the study showed a detection limit for B4/B5 and JPF/JPR primers pairs of 10 to 100 fg and 25 to 250 fg, respectively while the sensitivity for P1/P2 and 26A/26B primers pairs was of 12.5 to 125 fg and 20 to 200 fg respectively. All assays had also an excellent diagnostic sensitivity ranging from 95.5 to 100% in acute infection, depending on the PCR assay and the type of specimen. As blood is known containing inhibitory substances for PCR, the PCR detection limit was investigated [48] testing four primers pairs including B4-B5, ISp1-ISp2, F4-R2, JPF-JPR and modifying the previously reported methods [45,46]. Results indicated that the
Several multiplex PCRs which identify the genus Brucella at the species level and partially at the biovar level using different primer combinations, have been reported. The first multiplex PCR, called AMOS PCR assay (AMOS is an acronym from “abortus-melitensis-ovis-suis”), comprised five oligonucleotide primers for the identification of selected biovars of four species of Brucella. The assay exploited the polymorphism arising from species-specific localization of the genetic element IS711 in the Brucella chromosomes. Identity was determined by the size of the product amplified from primers hybridizing at various distances from the element. This method could identify three biovars (1, 2, and 4) of B. abortus, all three biovars of B. melitensis, all B. ovis biovars and biovar 1 of B. suis. An abbreviated multiplex AMOS PCR assay based on three additional primers was developed to differentiate B. abortus vaccine strain S19 and RB51 from field strains [50]. In 2005 the finding of a deletion next to one of the IS711 copies in B. abortus biovars 5, 6, 9 and in some field strains of biovars 3 of B. abortus has allowed to design and add a specific primer to the eight primer mixtures of AMOS PCR, allowing to enhance the discrimination power of this assay [51]. A RAPD-PCR (random amplified polymorphic DNA) was used in order to develop a multiplex PCR that uses the AMOS primers, additional specific loci of the insertion element IS711, and other unique insertions and deletions. This novel PCR assay differentiates between all presently recognized Brucella species, including the recently described species B. ceti (formerly named ‘Brucella maris’ or ‘Brucella cetaceae’), B. pinnipediaalis (formerly named ‘Brucella maris’ or ‘Brucella pinnipediaalis’), and B. microti, including some more recently described strains of the latter species [7-9], and also allows accurate differentiation of certain biovars of B. abortus and B. suis [52]. A new generation of multiplex PCR assays has been developed on the basis of the knowledge arisen from the recent availability of genome data. Garcia-Yoldi et al. [53] described a multiplex PCR assay for the identification of all six classical species, Brucella isolates from marine mammals, the vaccine strains B. abortus RB51 and S19 and B. melitensis Rev 1. The eight species-specific primer pairs amplified fragments of different sizes that showed a unique profile for each species following agarose gel electrophoresis. However, this multiplex PCR was unable to differentiate B. microti from B. suis and B. ceti from B. pinnipediaalis. A similar multiplex approach based on species-specific differences was recently described as being able to distinguish the six classical species but with some problems with B. canis and B. suis differentiation [49]. In addition some single target PCRs have proven particularly useful e.g. the presence of an insertion sequence, IS711, downstream of the bp26 gene, a feature specific to the marine mammal Brucella strains [54].

An advancement of the Garcia-Yoldi protocol for the differentiation of all currently described Brucella species was published by Mayer-Scholl et al. [55]. The primer pair identifying B.microti [7] was included in the multiplex PCR described by Garcia-Yoldi et al. [53], and the assay was set up on the DNA of Brucella reference strains and field isolates. The assay allowed the identification of all currently known Brucella, distinguishing also between the marine species B. ceti and B. pinnipediaalis and identifying the recently described species B. microti and B. inopinata.

### Real-time PCR

Real-time PCR is more rapid and more sensitive than conventional PCR. It does not require post amplification handling of PCR products, thereby reducing the risk of laboratory contamination and false-positive results. Real-time PCR assays have been recently described in order to test Brucella cells [56], urine [57], blood, and paraffin-embedded tissues [58]. Three separate real-time PCRs were developed to specifically identify seven biovars of B. abortus, three biovars of B. melitensis and biovar one of B. suis using fluorescence resonance energy transfer [56]. The upstream primers used in these real-time PCRs derived from the insertion element, IS711 whereas the reverse primer and FRET probes are selected from unique species or biovar-specific chromosomal loci. Sensitivity of B. abortus-specific assay was as low as 0.25 pg DNA corresponding to 16-25 genome copies and similar detection levels were also observed for B. melitensis and B. suis-specific assays. Light Cycler real-time PCR with SYBR Green 1 targeting bcsP31, a gene found in all Brucella species and biovars, was described [57]. The assay was per formed on DNA extracted by urine samples and showed a sensitivity of 10 fg corresponding to one genome copy. Another real-time PCR assay for the rapid laboratory diagnosis of human brucellosis on whole blood and paraffin-embedded tissues was developed [58] using three assays with hybridization probe detection. These assays targeted conserved and specific regions of the Brucella genome: the ribosomal 16S–23S ITS region, omp25 and omp31. The ITS-PCR clinical specificity was 100% and showed a limit of detection as low as 3 genome copies per reaction while omp25 and omp31 assays targeting only a single copy gene. Various molecular techniques differentiating Brucella at the species level and/or at the biovar level have been described [53,59-61]. These methods are usually less labour-intensive, faster than biochemical typing but these techniques did not set up with the aim of obtaining clear-cut species and biovar assignment in a very short time for routine laboratory testing. A real-time S’ nuclease PCR assay specific for amplification of a 322 bp fragment of the per osamine synthetase (per ) gene, a highly conserved region present in the naturally rough Brucella species B. ovis and B. canis and spontaneously rough strains of B. abortus and B. melitensis [54], was described by Bogdanovich et al. [62]. The assay showed a detection limit ranging from 200 fg (approximately 40 CFU) to 2 pg (approximately 400 CFU) but the differences could be due to the different DNA template quality obtained from different sources. The per forms of newly designed real-time PCR assays using TaqMan probes and targeting the 3 following specific genes: (i) the insertion sequence IS711, (ii) bcsP31 and (iii) per genes for the detection of Brucella at genus level was described by Bounaadja et al. [63]. The study showed that the use of the IS711-based TaqMan real-time PCR assay was specific, with the sensitivity 10 times higher of the two other targets, efficient and reproducible method for the rapid and safe detection of the genus Brucella. The bcsP31 and per targets could be used as confirmatory tools, in order to optimise the diagnostic specificity.
High resolution melt

The development of a molecular technique which utilizes real-time PCR followed by high-resolution melt (HRM) curve analysis to reliably type members of this genus has been described by Winchell et al. [64]. The assay targeted discriminating loci within the genomes of *Brucella* spp and through the dissociation curve analysis allowing the accurately identification of *Brucella* isolates at the species level and of unusual *Brucella* isolates such as BO1 and BO2. This assay also proved successful for discriminating *B. suis* from *B. canis*, but was unable to accurately differentiate a *B. suis* bv4 from *B. canis*. However, this particular *B. suis* biovar has previously been reported to exhibit a genotypic pattern identical to *B. canis*, and it is still debated as to whether this is truly a unique biovar of *B. suis* [65,66].

Restriction fraction length polymorphism (RFLP) based approaches

Recently, PCR-RFLP has provided evidence of polymorphism in a number of genes including the outer membrane protein 2 (omp2), the heat shock protein dnaK, htr, and the erythrolulose-1-phosphate dehydrogenase gene (ery). Particularly, the DNA polymorphism in omp2a, omp2b, omp25 and omp31 has been found to be useful for the differentiation between the *Brucella* species and their biovars, including the marine mammal *Brucella* isolates [59,67-71]. Results of PCR-RFLP allowed to identify in omp25 a marker for *B. melitensis* in the form of absence of an EcoRV site though *B. suis* biovars 3 and 4 and *B. canis* could still not be distinguished [70]. Other omp genes examined include omp31, known to be deleted in *B. abortus* [72], but which has markers for *B. canis*, *B. suis* biovar 2 and *B. ovis* [73].

Single nucleotide polymorphisms (SNPs) typing

Single nucleotide polymorphisms (SNPs) represent powerful markers that allow accurately describing the phylogenetic framework of a species, particularly in a genetically conserved group as *Brucella*. The approach is based on a series of discrimination assays interrogating SNPs that shown to be specific to a particular *Brucella* species. Scott et al. [74] described the use of SNPs in order to develop a multiplex SNP detection assay, based on primer extension technology, that can rapidly and unambiguously identify an isolate as a member of one of the six classical *Brucella* species or as a member of the recently identified marine mammal group. An alternative approach based on Minor Groove Binding protein (MGB) probes applied on a real-time PCR platform was described [75,76]. The assay distinguishes all members of the classical species, but the differentiation of *B. suis* and *B. canis* was difficult as no *B. suis* specific SNP has been identified. However, as a specific *B. canis* SNP has been identified [65], it is possible a discrimination with *B. suis*/*B. canis* specific SNP and the *B. canis* specific SNP [2]. A new SNP signatures for the rapid identification and biovar characterization of *B. suis* was described by Fretin et al. [77]. Allelic profiles unique for each *B. suis* biovar were defined and the most relevant signatures were determined. Biovars assigned with both present and classical methods were globally consistent except for some biovar 3 field strains which matched the allelic profile of biovar 1. An advancement of this method has represented by a novel SNP-based typing platform that, incorporating targets that define the three *Brucella* vaccine strains, allows the differentiation of the live *Brucella* vaccine strains from field isolates [78].

MALDI-TOF-MS spectra (MS spectra)

Bacterial identification based on peptidic spectra obtained by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry was proposed 30 years ago [79]. This method represents a new diagnostic tool in established microbiological laboratories [80]. Databases have been developed that include the main pathogenic microorganisms, thus allowing the use of this method in routine bacterial identification from plate culture. Recently, to identify *Brucella* species a reference library was constructed using 12 *Brucella* strains. With this 'Brucella library' discrimination was not possible to the species level [81].

Tandem repeat based typing (MLVA)

In the last years the availability of microbial genome sequences has facilitated the development of multilocus sequence-based typing approaches such as multiple locus VNTR (variable number of tandem repeats) analysis (MLVA). The VNTR, allelic hyper variability related to variation in the number of tandemly repeated sequences observed at several genomic loci in the *Brucella* genomes, were used for the discrimination of bacterial species that display very little genomic diversity. The first application of VNTR based typing to *Brucella* was the HOOF-Prints scheme (Hyper variable Octomeric Oligonucleotide Finger-Prints) published by Bricker [82]. The approach was based on a comparison of the newly completed genome sequences of *B. suis* and *B. melitensis* along with a draft *B. abortus* sequence which identified an eight base pair tandem repeat sequence at nine distinct genomic loci [2]. Eight of the nine loci were variable among the three genome sequences allowing the development of a PCR-based method to identify the number of repeat units at each locus. HOOF-Prints have been employed with entire genome sequences to identify rapidly evolving loci and are capable of differentiating *Brucella* isolates by the variability in the 8-bp tandem repeat [82]. HOOF-Prints has been used to investigate the clustering of *Brucella* isolates [83,84]. In 2006 Whatmore et al. [85] described a new scheme that included the eight of the original loci of Bricker as well as an additional 13 newly VNTR loci to give a 21 locus scheme, VNTR-21, that allowed to provide some resolution at the species level. In the same year a scheme labelled MLVA-15, based on a subset of 15 loci that comprises 8 markers with good species identification capability and 7 with higher discriminatory power, was published [86], and followed by MLVA-16, a slight modification of MLVA-15 [87]. MLVA16 has been used to recognise human outbreaks that relate to a common source or to confirm relapse [88] or for tracing back source of laboratory infection [89] as well as to demonstrate heterogeneity in profiles even in a restricted area of endemicity [90] and identify that human *B. melitensis* isolates from *Per* *u* form a distinct cluster from previously described European isolates [91]. MLVA-15 has also been used to assess the stability of a live vaccine [92], to show that wild boar and domestic pigs sharing localities can have identical *B. suis* genotypes [93], identifies clusters that are congruent with species identification [94]. The MLVA band profiles, obtained by the amplification of the different alleles, may be resolved by different techniques ranging from low cost manual agarose gels to the more expensive capillary electrophoresis sequencing systems. Recently, a more rapid and inexpensive method based on the Lab on a chip technology has been proposed [95]. This miniaturized platform for electrophoresis applications is able to size and quantify PCR fragments, and was previously used for studying the genetic variability of *Brucella* spp. [96]. On the basis of this equipment a new high throughput microfluidics system, the LabChip 90 equipment (Caliper Life Sciences), applied to the selected subset of 16 loci proposed by Al-Dahouk [87] for MLVA typing of *Brucella* strains was developed [97].
Conclusion

Since Amerithrax incident in 2001, when the deliberate release of anthrax spores via mail within USA caused five deaths and panic spreads across North America, the governments focused on the enhancing of their biosecurity and biosafety programs. It was also highlighted the requirement for the acquisition of new technologies in order to detect and differentiate the biological warfare agents. Particularly, the need for discriminating between natural outbreaks or deliberate microorganism release brought to the upgrowth of new genotyping methods. Although Brucella represents more a biological warfare agent of historical significance than a real threat, it is also the etiologic agent of one of the world’s major zoonotic infections, responsible for economic losses and considerable human morbidity [14,98]. Therefore, the world community was motivated to strengthen the control mechanisms for this agent. Since the first signs of a bioterrorism attack could appear in the same way of a natural outbreak, the development of methods for detection and identification of biological warfare agents has been considered a need by many countries, though the genetic homogeneity of the genus hamper s this target. For the direct detection, speciation and differentiation of Brucella spp recovered from clinical and environmental specimens, in the last years different target genes, primer pairs, PCR techniques and extraction procedures have been proposed. Further standardisation and optimisation are necessary to achieve consistency and reliability of results before they are incorporated in routine laboratory investigations because of differences of sensitivity of the assays, due to different DNA extraction methods, detection formats and limits, and to different types of specimens used. Moreover, the development of DNA technologies for the molecular subtyping of Brucella, have been described; for example over the last years whole-genome analysis provided new insights into this genus. The first Brucella genome sequence available was B. melitensis 16 M [99], followed by B. suis 1330 [100], B. abortus 2308 and 9-941 [101,102], the full sequence of the vaccine strain B. abortus S19 [103], B. canis, B. ovis, B. suis biovar 2, B. melitensis biovar 2 and an incomplete B. ceti genome [104] as well as genomes of B. suis biovar 3 and 4 and B. melitensis biovar 3 [105]. At this time, about forty genome sequences from different Brucella strains, representing all species, have been published either as complete genomes [106,107], or as draft assemblies in the complete genome and whole genome shotgun divisions of GenBank NCBI (National Center for Biotechnology Information. http://www.ncbi.nlm.nih.gov), or can be downloaded from the Pathosystems Resource Integration Center web site (http://brcdownloads.vbi.vt.edu/patric2/PATRIC/). The genomic analysis allowed revealing the genetic divergence within Brucella spp. as the variable number tandem repeat sequences throughout the genome, hyper variability due to recombination between and within the repeats. MLVA represents the most used approach allowing distinction between genetically homogenous species, as well as the close genetic relationship represented by biosvars within a species or clade. The development of MLVA based methods on high throughput capillary gel electrophoresis or microfluidics technology provides robust high-resolution typing tools for epidemiological trace-backing, suitable for high throughput analysis and inter-laboratory comparisons, offering a fair compromise among costs, speed and specificity, compared to any of the conventional molecular typing techniques. More recent advances in sequencing technology produced a new class of massively parallel next-generation sequencing platforms such as: Illumina, Inc. Genome Analyzer, Applied Biosystems Solid System, and 454 Life Sciences (Roche) GS FLX that could represent a promising epidemiological and forensic typing tools. However, the advent of bioinformatics, genome-sequencing and high-throughput genome-wide platforms has lead to an impressive enhancement of the data flow. Therefore the storing and the integration of these heterogeneous informations have become the crucial point to facilitate data exchange, analysis and identification of suspect agent. Bioinformatics programs have been adopted by several research centres in order to provide a wide integration of genomic, transcriptomic and proteomic data, which is essential for developing a system-centric resource needed for supporting the research or forensic investigations. These programs allow the storage of data, providing visualization and analysis tools. Furthermore, there are publicly available databases for storing and disseminating data, as MLVAbank for Bacterial Genotyping [108] which works as repositories of individual data types.

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