High throughput MLVA-16 typing for Brucella based on the microfluidics technology

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Abstract

Background: Brucellosis, a zoonosis caused by the genus Brucella, has been eradicated in Northern Europe, Australia, the USA and Canada, but remains endemic in most areas of the world. The strain and biovar typing of Brucella field samples isolated in outbreaks is useful for tracing back source of infection and may be crucial for discriminating naturally occurring outbreaks versus bioterrorist events, being Brucella a potential biological warfare agent. In the last years MLVA-16 has been described for Brucella spp. genotyping. The MLVA band profiles may be resolved by different techniques i.e. the manual agarose gels, the capillary electrophoresis sequencing systems or the microfluidic Lab-on-Chip electrophoresis. In this paper we described a high throughput system of MLVA-16 typing for Brucella spp. by using of the microfluidics technology.

Results: The Caliper LabChip 90 equipment was evaluated for MLVA-16 typing of sixty-three Brucella samples. Furthermore, in order to validate the system, DNA samples previously resolved by sequencing system and Agilent technology, were de novo genotyped. The comparison of the MLVA typing data obtained by the Caliper equipment and those previously obtained by the other analysis methods showed a good correlation. However the outputs were not accurate as the Caliper DNA fragment sizes showed discrepancies compared with real data and a conversion table from observed to expected data was created.

Conclusion: In this paper we described the MLVA-16 using a rapid, sophisticated microfluidics technology for detection of amplification product sizes. The comparison of the MLVA typing data produced by Caliper LabChip 90 system with the data obtained by different techniques showed a general concordance of the results. Furthermore this platform represents a significant improvement in terms of handling, data acquiring, computational efficiency and rapidity, allowing to perform the strain genotyping in a time equal to one sixth respect to other microfluidics systems as e.g. the Agilent 2100 bioanalyzer. Finally, this platform can be considered a valid alternative to standard genotyping techniques, particularly useful dealing with a large number of samples in short time. These data confirmed that this technology represents a significative advancement in high-throughput accurate Brucella genotyping.

Background

The members of the genus Brucella are Gram-negative, facultative intracellular bacteria responsible of a considerable human morbidity and in animals of enormous economic losses [1] due to abortion and infertility in livestock (cattle, goats, and sheep). As brucellosis is a zoonotic disease, practically all human Brucella infections develop from direct or indirect contact to animals.

In particular, brucellosis in humans occurs as a sub-acute or chronic illness, that 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 initiated [2]. There are nine recognized species of Brucella [3] that differ in their host preference [4]. In particular, the nine recognized host-specific Brucella spp. are: B. abortus which preferentially infects cattle; B. melitensis infects sheep and goats; B. suis infects pigs; B. canis the dog; B. ovis, sheep and goats; B. neotomae the desert wood rat; B. microti the common vole [5]; B. ceti, cetaceans [6];
B. pinnipedialis, seals [6,7]. Recently, an additional novel species, B. inopinata sp., isolated from a human breast implant infection, was described [8]. Currently, the division in species and between biovars of a given species is performed using differential tests based on phenotypic characterizations of lipopolysaccharide (LPS) antigens, phage typing, dye sensitivity, requirement for CO₂, H₂S production, and metabolic properties [9]. The genotyping of Brucella field strain isolated in outbreaks is an essential tool to better understand the epidemiology of the disease and to give support to the trace-back of infection sources. It is also essential to identify the presence of Brucella strains that can affect livestock populations and new strains that were previously considered to be exotic [10], thus improving the outcomes of the national brucellosis eradication programme. Although brucellosis has been eradicated in Northern Europe, Australia, the USA and Canada, this disease remains endemic in most areas of the world [11]. Therefore, the knowledge of the prevailing genotypes of Brucella spp. present in a country is an important epidemiological tool to assess the necessary steps required for the formulation of policies and strategies for the control of brucellosis in animal populations. In addition, Brucella spp. represent potential biological warfare agents due to the high contagious rates for humans and animals, the non-specific symptoms associated with the infection, and the fact that the organism can be readily aerosolized [12-14]. Therefore, the discrimination between natural outbreaks and/or intentional release of micro-organism agents may be of crucial importance in the context of the bioterrorism. Brucella species are characterised by >80% interspecies homology by DNA-DNA hybridization studies [15,16] and >98% sequence similarity by comparative genomics [17]. In fact, the sequencing of 16 S rRNA showed a 100% of identity between all of the Brucella spp. [18]. The simple identification of genus and, in some cases, species by PCR assays [19,20], is adequate for purposes as diagnosis of human/animal disease or identification of food contamination but not for the tracing of outbreaks or bioterrorist attacks. Therefore, the development of strain typing methods is essential in order to investigate the source of an epidemic event. Molecular DNA technology such as repetitive intergenic palindromic sequence-PCR (REP-PCR) [21], random amplified polymorphic DNA-PCR (RAPD-PCR) [22], arbitrary primed-PCR (AP-PCR) [23], amplified fragment length polymorphism (AFLP) [24], single nucleotide polymorphism (SNP) [25,26], and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) [27] has been employed to sub-type Brucella spp.

In the last years the variable number of tandem repeats (VNTR), allelic hypervariability related to variation in the number of tandemly repeated sequences, were used for the discrimination of bacterial species that display very little genomic diversity. Polymorphic tandem repeat loci have been identified by analysing published genome sequences of B. melitensis 16 M, B. suis 1330, and B. abortus 9-941 [16,28]. Schemes based on multiple locus VNTR analysis (MLVA) were tested. In Brucella, MLVA schemes with 21 loci (MLVA-21), 15 and 16 loci (MLVA-15 and MLVA-16) were published [12,16,29]. The authors used a subset of loci that preserved the clusters corresponding to classical species, comprising markers with repeat unit sizes of 9 bp or greater and good species identification capability (‘minisatellites’) and markers with repeat unit sizes of up to 8 bp and higher discriminatory power (‘microsatellites’) [30]. The MLVA band profiles may be resolved by different techniques ranging from low cost manual agarose gels to the more expensive capillary electrophoresis sequencing systems. The most frequently used method is the agarose gel. Recently, a more rapid and inexpensive method based on the Lab-on-a-chip technology has been proposed [31]. 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. [32]. Recently a high throughput micro-fluidics system, the LabChip 90 equipment (Caliper Life Sciences), was developed. This platform can be considered particularly useful when dealing with a large number of samples in short time. Therefore we evaluated the LabChip 90 system for MLVA typing of Brucella strains applying the selected subset of 16 loci proposed by Al-Dahouk et al. [12] to fifty-three field isolates and ten DNA samples provided in 2006 for Brucella suis ring-trial. Furthermore, twelve DNA samples, provided in 2007 for a MLVA VNTR ring trial and seventeen human Brucella isolates whose MLVA fingerprinting profiles were previously resolved [32,33], were de novo genotyped.

Results
By means of MLVA-16 on LabChip 90 (Caliper Life Sciences) sixty-three DNA samples, fifty-three field isolates of Brucella (Table 1) and ten DNA provided for Brucella suis ring-trial, were analysed for investigating a broader number of loci. In order to set up the system, DNA samples, previously genotyped by sequencing system and Agilent technology [32,33], were reanalyzed. DNA from all ninety-two isolates was amplified at 16 loci (MLVA-16 typing assay) to generate multiple band profiles. The LabChip 90 equipment acquires the sample in less than a minute and the analysis of 96 samples in less than an hour. After PCR amplification 5 μl of each reaction was loaded into a 96-well plate and the amplification product size estimates were obtained by the LabChip Gx Software. The data produced by the Caliper
Table 1 The fifty-three strains provided by Istituto Zooprofilattico Sperimentale dell’Abruzzo e del Molise - G. Caporale-(Istituto G. Caporale)

| Samples   | Species-biovar according MLVA Database Genotyping | Year | Host     | Geographic origin |
|-----------|---------------------------------------------------|------|----------|-------------------|
| BruIT200  | B.melitensis biovar 3                             | 2002 | human    | Sardinia, Italy   |
| BruIT201  | B.abortus biovar 1                                | 2002 | bovine   | Piemonte, Italy   |
| BruIT202  | B.melitensis biovar 3                             | 2002 | bovine   | Lazio, Italy      |
| BruIT203  | B.abortus biovar 1                                | 2002 | bovine   | Lazio, Italy      |
| BruIT204  | B.abortus biovar 3                                | 2002 | bovine   | Piemonte, Italy   |
| BruIT205  | B.melitensis biovar 3                             | 2002 | water buffalo | Campania, Italy |
| BruIT206  | B.melitensis biovar 3                             | 2002 | water buffalo | Campania, Italy |
| BruIT207  | B.abortus biovar 1                                | 2003 | water buffalo | Campania, Italy |
| BruIT208  | B.melitensis biovar 3                             | 2003 | milk     | Emilia-Romagna, Italy |
| BruIT209  | B.melitensis biovar 3                             | 2003 | bovine   | Abruzzo, Italy    |
| BruIT210  | B.abortus biovar 3                                | 2001 | bovine   | Piemonte, Italy   |
| BruIT211  | B.abortus biovar 3                                | 2001 | bovine   | Piemonte, Italy   |
| BruIT212  | B.abortus biovar 3                                | 2002 | bovine   | Piemonte, Italy   |
| BruIT213  | B.abortus biovar 3                                | 2007 | bovine   | Italy             |
| BruIT214  | B.abortus biovar 3                                | 2002 | bovine   | Piemonte, Italy   |
| BruIT215  | B.melitensis biovar 3                             | 2001 | ovine    | Lazio, Italy      |
| BruIT216  | B.melitensis biovar 3                             | 2001 | ovine    | Lazio, Italy      |
| BruIT217  | B.melitensis biovar 3                             | 2001 | water buffalo | Lazio, Italy |
| BruIT218  | B.melitensis biovar 3                             | 2002 | bovine   | Campania, Italy   |
| BruIT219  | B.melitensis biovar 3                             | 2001 | wild boar | Campania, Italy   |
| BruIT220  | B.melitensis biovar 3                             | 2001 | bovine   | Piemonte, Italy   |
| BruIT221  | B.melitensis biovar 3                             | 2001 | ovine    | Piemonte, Italy   |
| BruIT222  | B.melitensis biovar 3                             | 2001 | ovine    | Lazio, Italy      |
| BruIT223  | B.melitensis biovar 3                             | 2001 | ovine    | Lazio, Italy      |
| BruIT224  | B.abortus biovar 3                                | 2001 | bovine   | Lazio, Italy      |
| BruIT225  | B.abortus biovar 3                                | 2001 | bovine   | Piemonte, Italy   |
| BruIT226  | B.melitensis biovar 3                             | 2001 | human    | Lazio, Italy      |
| BruIT227  | B.suis biovar 2                                   | 2003 | hare     | Emilia-Romagna, Italy |
| BruIT228  | B.suis biovar 2                                   | 2003 | hare     | Emilia-Romagna, Italy |
| BruIT239  | B.abortus biovar 3                                | 2008 | bovine   | Molise, Italy     |
| BruIT240  | B.abortus biovar 3                                | 2008 | bovine   | Molise, Italy     |
| BruIT241  | B.abortus biovar 3                                | 2008 | bovine   | Molise, Italy     |
| BruIT242  | B.abortus biovar 3                                | 2008 | bovine   | Molise, Italy     |
| BruIT243  | B.abortus biovar 3                                | 2008 | bovine   | Molise, Italy     |
| BruIT244  | B.abortus biovar 3                                | 2008 | bovine   | Molise, Italy     |
| BruIT245  | B.abortus biovar 3                                | 2007 | water buffalo | Campania, Italy |
| BruIT246  | B.melitensis biovar 3                             | 2007 | water buffalo | Campania, Italy |
| BruIT247  | B.abortus biovar 3                                | 2007 | bovine   | Calabria, Italy   |
| BruIT248  | B.abortus biovar 3                                | 2007 | water buffalo | Puglia, Italy |
| BruIT249  | B.abortus biovar 3                                | 2009 | bovine   | Campania, Italy   |
| BruIT250  | B.abortus biovar 3                                | 2009 | bovine   | Calabria, Italy   |
| BruIT251  | B.abortus biovar 3                                | 2009 | bovine   | Calabria, Italy   |
| BruIT252  | B.abortus biovar 6                                | 2009 | bovine   | Calabria, Italy   |
| BruIT253  | B.abortus biovar 6                                | 2009 | ovine    | Puglia, Italy     |
| BruIT254  | B.melitensis biovar 3                             | 2001 | bovine   | Piemonte, Italy   |
| BruIT255  | B.abortus biovar 3                                | 2002 | bovine   | Piemonte, Italy   |
| BruIT256  | B.suis biovar 2                                   | 2002 | bovine   | Piemonte, Italy   |
| BruIT257  | B.suis biovar 2                                   | 2001 | ovine    | Lazio, Italy      |
| BruIT258  | B.suis biovar 2                                   | 2005 | water buffalo | Campania, Italy |
| BruIT259  | B.suis biovar 2                                   | 2002 | wild boar | Piemonte, Italy   |
system showed band sizing discrepancies compared with data obtained from other electrophoresis platforms. Therefore a conversion table that would allow the allocation of the correct alleles to the range of fragment sizes was created. The table contained for each locus the expected size, the range of observed sizes, including arithmetical average ± standard deviation, and the corresponding allele (Table 2). The variability range for each allele was established experimentally by the analysis of different strain amplification products. Furthermore, in order to look at intra- and interchip variability, each allele was analyzed by repeating five times the analysis on the same chip and different chips. The comparison of the average and standard deviations obtained by the analysis of the intra- and interchip variability by t-test (confidence of interval 95%) shown a P value > 0.005 (data not shown).

The required precision is directly related to the repeat unit size of the loci. Only data with a standard deviation lower than the 50% of the repeat unit size were considered valid. The LabChip 90 equipment MLVA-16 products were separated and DNA fragment sizes were correlated to the alleles by the conversion table. Generally, close alleles were not observed to overlap allowing to assign the correct allele to each observed value. However, the markers Bruce 08, Bruce 21, Bruce 16 and Bruce 19 showed continuity between some neighboring range which may lead to incorrect assignment of allele to the observed value (Table 2). The identified species were compared with the results of the previous analysis [32,33], obtaining a full concordance for 15 markers while the marker Bruce 19 did not show agreement with the results obtained by the different analysis systems. For the loci including alleles spanning into ambiguous ranges, we performed sequencing of the amplicons showing on Caliper maximum or minimum allele values. Furthermore we performed some random sequencing of the amplicons obtaining a confirmation of the correct assignment (data not shown).

**Discussion**

Many methods have been developed to differentiate *Brucella* strains but MLVA currently represents one of the most promising technologies regarding the epidemiology of bacteria with a high genetic homogeneity, such as *Brucella* ssp. In 2003 Bricker et al [28] published a MLVA based on eight locus scheme. In 2006 Whatmore et al [16] 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 [29], and followed by MLVA-16, a slight modification of MLVA-15 [12]. The different alleles, amplified by standard PCR techniques, can be analysed by several electrophoretic techniques as agarose gel, or capillary electrophoresis sequencing. In this paper the attention was addressed on the LabChip 90 equipment (Caliper), a platform based on microfluidics technology specifically developed for measuring the length of DNA fragments and that do not require fluorescent primers. This electrophoresis machine represents a compromise between the more expensive capillary electrophoresis apparatus and the traditional agarose gel electrophoresis. In spite of a lower precision respect to the automated capillary electrophoresis, the ability to acquire 96 amplification product sizes in less than a hour represent an increased time-reduction over the traditional ethidium bromide slab gel electrophoresis, with 40-50 amplification product sizes for the same analysed markers acquired in a higher time [34]. The LabChip 90 represents also a significant improvement respect to other microfluidics systems as e.g. the Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, Ca). In effect the LabChip 90 allows performing the strain genotyping in a time equal to one sixth respect to Agilent. Furthermore this system requires less handling as a single plate can be read directly after the PCR reaction, while the Agilent equipment needs a manual charge of the single PCR products for each single chip well. Finally, the LabChip GX software improves efficiency of data acquiring by automating the data flows. In fact, the software allows to export the summary of analysis results to a spreadsheet application, with the consequent elimination of the paper-based flows. As described previously [31,32] the sizing proposed by the Lab on chip technology does not correspond to the real size, resulting in a shift of a variable value (offset) respect to the real size estimated by sequencing. Therefore, a correspondence table which allows for each range of observed values to assign the expected size and corresponding allele (Table 2) was created. We did not observe in general the overlap among
Table 2 Comparison between *Brucella* product sizes estimated by LabChip GX software (Observed size) and actual sizes obtained by direct sequencing of the PCR product or data available in Genbank (Expected size)

| PCR          | Locus (UL bps)a | Allele | Expected size | Observed size | x ± s b |
|--------------|-----------------|--------|---------------|---------------|---------|
| Singleplex 1 | Bruce08 (18)    | 2      | 312           | 320           | 352,63 ± 5,37 |
|              |                 | 3      | 330           | 346-359       | 369,38 ± 4,62 |
|              |                 | 4      | 348           | 369-383       | 385,410 ± 6,58 |
|              |                 | 5      | 366           | 411-434       | 419,29 ± 6,71 |
|              |                 | 6      | 384           | 407           | 409,420 ± 2,32 |
| Singleplex 2 | Bruce43 (12)    | 1      | 170           | 179-188       | 183,17 ± 2   |
|              |                 | 2      | 182           | 191-200       | 196,07 ± 2,32 |
|              |                 | 3      | 194           | 197-206       | 199,57 ± 2,13 |
| Singleplex 3 | Bruce12 (15)    | 7      | 302           | 359-369       | 362,8 ± 3,7 |
|              |                 | 8      | 317           | 379-388       | 384,13 ± 3,64 |
|              |                 | 9      | 332           | 390-400       | 395,16 ± 3,05 |
|              |                 | 10     | 347           | 409-420       | 413,25 ± 2,87 |
|              |                 | 11     | 362           | 424-433       | 428,82 ± 3,05 |
|              |                 | 12     | 377           | 434-440       | 438,25 ± 2,87 |
| Singleplex 4 | Bruce18 (8)     | 3      | 130           | 143           | 153,57 ± 2,64 |
|              |                 | 4      | 138           | 150-157       | 160,33 ± 1,37 |
|              |                 | 5      | 146           | 164-176       | 171,62 ± 2,95 |
|              |                 | 6      | 154           | 178-184       | 181,65 ± 1,53 |
|              |                 | 7      | 162           | 187-194       | 191 ± 2,24 |
| Singleplex 5 | Bruce11 (63)    | 2      | 257           | 266-270       | 268 ± 2,82 |
|              |                 | 3      | 320           | 321-344       | 337,82 ± 4,31 |
|              |                 | 4      | 383           | 407-422       | 410,52 ± 3,56 |
|              |                 | 6      | 509           | 504-536       | 515,8 ± 12,52 |
|              |                 | 8      | 635           | 623-649       | 639,6 ± 8,71 |
|              |                 | 9      | 698           | 680-724       | 696,67 ± 15,6 |
|              |                 | 12     | 887           | 187-194       | 191 ± 2,24 |
|              |                 | 15     | 1076          | 106-108       | 108,5 ± 2,11 |
| Singleplex 6 | Bruce21 (8)     | 5      | 140           | 141           | 141,57 ± 2,64 |
|              |                 | 6      | 148           | 162           | 162,5 ± 2,11 |
|              |                 | 7      | 156           | 178-199       | 182,55 ± 1,19 |
|              |                 | 8      | 164           | 182-199       | 194,05 ± 1,94 |
| Singleplex 7 | Bruce06 (134)   | 1      | 140           | 151           | 151 ± 3,13 |
|              |                 | 2      | 274           | 282-294       | 285,9 ± 3,33 |
|              |                 | 3      | 408           | 429-454       | 439,89 ± 6,04 |
|              |                 | 4      | 542           | 518-624       | 575,4 ± 24,92 |
| Singleplex 8 | Bruce42 (125)   | 1      | 164           | 172-198       | 175 ± 3,13 |
|              |                 | 2      | 289           | 279-298       | 288,88 ± 2,14 |
|              |                 | 3      | 414           | 420-442       | 428,27 ± 6,18 |
|              |                 | 4      | 539           | 504-569       | 529,31 ± 14,1 |
|              |                 | 5      | 664           | 642-647       | 644 ± 2,64 |
|              |                 | 6      | 789           | 695-763       | 726,4 ± 22,02 |
|              |                 | 7      | 914           |               |              |
| Singleplex 9 | Bruce45 (18) | 2   | 133 |
|            |              | 3   | 151 |
|            |              | 4   | 169 |
|            |              | 5   | 187 |
| Singleplex 10 | Bruce55 (40) | 1   | 193 |
|            |              | 2   | 233 |
|            |              | 3   | 273 |
|            |              | 4   | 313 |
|            |              | 5   | 353 |
|            |              | 6   | 393 |
|            |              | 7   | 433 |
| Singleplex 11 | Bruce30 (8) | 2   | 119 |
|            |              | 3   | 127 |
|            |              | 4   | 135 |
|            |              | 5   | 143 |
|            |              | 6   | 151 |
|            |              | 7   | 159 |
|            |              | 8   | 167 |
|            |              | 9   | 175 |
|            |              | 10  | 183 |
|            |              | 11  | 191 |
|            |              | 12  | 199 |
| Singleplex 12 | Bruce04 (8) | 2   | 152 |
|            |              | 3   | 160 |
|            |              | 4   | 168 |
|            |              | 5   | 176 |
|            |              | 6   | 184 |
|            |              | 7   | 192 |
|            |              | 8   | 200 |
|            |              | 9   | 208 |
|            |              | 10  | 216 |
|            |              | 11  | 224 |
|            |              | 12  | 232 |
|            |              | 13  | 240 |
|            |              | 14  | 248 |
|            |              | 15  | 256 |
|            |              | 17  | 272 |
|            |              | 18  | 280 |
|            |              | 19  | 288 |
|            |              | 20  | 296 |
|            |              | 22  | 312 |
| Singleplex 13 | Bruce07 (8) | 2   | 134 |
|            |              | 3   | 142 |
|            |              | 4   | 150 |
|            |              | 5   | 158 |
|            |              | 6   | 166 |
|            |              | 7   | 174 |
|            |              | 8   | 182 |
|            |              | 9   | 190 |
|            |              | 10  | 198 |
|            |              | 11  | 206 |
Table 2 Comparison between *Brucella* product sizes estimated by LabChip GX software (Observed size) and actual sizes obtained by direct sequencing of the PCR product or data available in Genbank (Expected size) (Continued)

| Singleplex 14 | Bruce 09 (8) |     | 131-140 | 135.52 ± 2.6 |
|---------------|--------------|-----|---------|--------------|
| 12            | 214          |     |         |              |
| 13            | 222          |     |         |              |
| 14            | 230          |     |         |              |

| Singleplex 15 | Bruce 16 (8) |     | 153-157 | 154.9 ± 1.59 |
|---------------|--------------|-----|---------|--------------|
| 2             | 144          |     |         |              |
| 3             | 152          |     |         |              |
| 4             | 160          |     |         |              |
| 5             | 168          |     |         |              |
| 6             | 176          |     |         |              |
| 7             | 184          |     |         |              |
| 8             | 192          |     |         |              |
| 9             | 200          |     |         |              |
| 10            | 208          |     |         |              |
| 11            | 216          |     |         |              |
| 12            | 224          |     |         |              |
| 13            | 232          |     |         |              |
| 14            | 240          |     |         |              |
| 15            | 248          |     |         |              |

| Singleplex 16 | Bruce 19 (6) |     | 173-177 | 175 ± 1.4  |
|---------------|--------------|-----|---------|------------|
| 4             | 79           |     |         |            |
| 5             | 85           |     |         |            |
| 6             | 91           |     |         |            |
| 15            | 145          |     |         |            |
| 16            | 151          |     |         |            |
| 18            | 163          |     |         |            |
| 19            | 169          |     |         |            |
| 20            | 175          |     |         |            |
| 21            | 181          |     |         |            |
| 22            | 187          |     |         |            |
| 23            | 193          |     |         |            |
| 25            | 205          |     |         |            |

*a* Unit Length size.

*b* Arithmetic average (x) ± standard deviation (s) of the observed sizes.
close alleles, allowing to unambiguously assign the correct allele to each observed value. However, for some contiguous alleles we observed a continuity between ranges which may lead to incorrect assignment of allele to the observed value (Table 2). Furthermore, the instrument was not in agreement with the results obtained by the different analysis systems for the marker Bruce 19. The reduced discriminatory ability could be due to the different resolution achieved by such platform related to the fragment sizes (routinely ± 10% in a 150-500 -bp range, ± 15% in a 100-150 -bp range and in a 500-1500 -bp range and ± 20% in a 1500-5000 -bp range). However, the comparison of the results obtained by the MLVA-16 method on the Caliper LabChip 90 platform and those previously resolved by capillary electrophoresis sequencing system and the Lab on a chip technology (Agilent Technologies) showed a good size correlation. Therefore, this platform can be considered a valid alternative to standard genotyping techniques, particularly useful dealing with a large number of samples in short time.

**Conclusion**

In this paper we evaluated high throughput system as the LabChip 90 for MLVA-16 typing of *Brucella* strains. The MLVA typing data obtained on this equipment showed accurate correlation for those obtained by capillary electrophoresis sequencing and the Agilent 2100 Bioanalyzer, with the exception of Bruce 19. This new platform represents a significant improvement of the genotyping techniques in terms of turnaround times and computational efficiency.

**Methods**

**Brucella** strains and DNA extraction

In this study fifty-three field isolates submitted for typing by the Istituti Zooprofilattici Sperimentali to the National Reference Laboratory for brucellosis at the Istituto Zooprofilattico Sperimentale dell’Abruzzo e del Molise-G. Caporale (Istituto G. Caporale) during the 2001-2008 period (Table 1), ten DNA samples, collected in UK, provided at the Istituto Zooprofilattico Sperimentale dell’Abruzzo e del Molise-G. Caporale (Istituto G. Caporale) for *Brucella suis* ring-trial 2006 (COST 845-Brucellosis in man and animals), seventeen *Brucella* strains isolated from Sicilian hospitalized patients with acute brucellosis [33], and twelve DNA samples, provided by Dr. Falk Melzer for the Ring trial *Brucella* 2007 [32], were analysed. The provided DNA samples were extracted by Maxwell 16 Cell DNA purification kit (Promega), according to the manufacturer’s instructions.

**VNTR amplification**

VNTR amplifications were performed according to the method described by Le Flèche et al. [29] and then adapted by Al Dahouk et al [12]. Sixteen sets of primers previously proposed were used in sixteen singleplex: Bruce06, Bruce08, Bruce11, Bruce12, Bruce42, Bruce43, Bruce45, Bruce55 (panel 1), Bruce18, Bruce 19, Bruce21, Bruce04, Bruce07, Bruce09, Bruce16, and Bruce30 (panel 2). Amplification reaction mixtures were prepared in 15 μL volumes using 1U FastStart polymerase Taq (Roche) and containing 1 ng of DNA, 1 × PCR Roche reaction buffer (10 mM Tris-HCl, 2.5 mM MgCl2, 50 mM KCl pH 8.3), 0.2 mM dNTPs (Roche) and 0.3 μM of each flanking primer. The amplification was performed in a Peltier Thermal Cycler DNA Engine DYAD (M Research) thermocycler as follows: an initial heating at 95°C for 5 min, 35 cycles denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec and extension at 70°C for 60 sec. A final extension was performed at 70°C for 5 min [32].

**MLVA-16 analysis**

The amplification was performed in 96-well or 384-well PCR plates. The chip was prepared according to manufacturer recommendations (Caliper HT DNA 5 K Kit). Each chip contains 5 active wells: 1 for the DNA marker and 4 for gel-dye solution. For each run it was prepared also a strip well with the ladder (containing eight MW size standards of 100 300 500 700 1100 1900 2900 4900 bp) that was inserted into the appropriate groove of the instrument. The number of samples per chip preparation is 400, equivalent or four 96-well plates or one 384-well plate. After gel preparation, the sample plate was loaded into the plate carrier attached to the robot of the Caliper LabChip 90. During the separation of the fragments, the samples were analyzed sequentially and electropherograms, virtual gel images and table data were shown. Amplification product size estimates were obtained by using the LabChip GX (Caliper Life Sciences). The software allows importing the data to a spreadsheet software and subsequently to the conversion table that, by a special macro set up by our laboratory, allows to assign each size to the corresponding allele. The maximum and minimum value of the observed sizes for each allele was thus established experimentally while the arithmetic average and the corresponding standard deviation (Table 2) were calculated by a statistical function.

**Sequencing analysis**

The PCR amplicons were purified and sequenced by CEQ 8000 automatic DNA Analysis System (Beckman-Coulter, Fullerton, CA, USA) using a commercial Kit (Genome-Lab™ DTCS-Quick Start Kit, Beckman-Coulter) according to the manufacturer instructions.

**Acknowledgements**

This work was part of the European Defence Agency (EDA) project 80060 involving bio-defence institutions from Sweden, Norway, the Netherlands, Germany, France and Italy.
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Authors' contributions
RDes and ACia did the set up of the Brucella MLVA-16 assay. Rdes, ACia and CMa participated to typing work. FL, EDG and MAN did the error checking analysis. SFl and GFA did various sequence analysis. FLG, RDes and RDes were in charge of the database and clustering analyses. FL, MAN, and RDes conceived the study. FL and RDes wrote the report. All authors read and approved the final manuscript.

Received: 8 October 2010 Accepted: 24 March 2011
Published: 24 March 2011

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