Short communication

“HOOF-Print” genotyping and haplotype inference discriminates among Brucella spp. isolates from a small spatial scale

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1. Introduction

Brucellosis is an important zoonosis with worldwide distribution and high clinical morbidity. Bacteria from the genus Brucella can infect a variety of hosts and are responsible for significant economic losses in livestock industries and serious public health problems in humans. Transmission to man can occur through many routes: foodborne, occupational, recreational and potentially through bioterrorism (Godfroid et al., 2005). Brucellosis continues to be a major problem in the Mediterranean Basin, Middle East, Latin America, Asia and Africa (Godfroid et al., 2005; Pappas et al., 2006). Portugal, like other European Union countries, employs specific regulations and measures to eradicate the disease. Regardless of the huge efforts to eliminate it, human cases still occur frequently in the country (16.1 cases per million, Direcção Geral de Saúde, Divisão de Epidemiologia, 2006) and the prevalence in cattle and small ruminants herds is 0.25 and 0.70%, respectively (Direcção Geral de Veterinária, 2007).

Control of brucellosis, particularly in the final stages of an eradication program, requires a rigorous program for surveillance and highly discriminatory methods for characterizing an outbreak strain, which can be used in trace back studies to determine the original source of infection and its routes of transmission. Conventional methods for subtyping of Brucella strains into species and biovars have some shortcomings, particularly, in small geographical regions where few biovars tend to predominate (e.g., most B. melitensis isolated in Portugal belong to biovars 1 and 3; LNIV, National Laboratory for Veterinary Research, unpublished data). Also, classification of Brucella relies on a large array of phenotypic tests that are prone to misinterpretation or inaccuracy (Banai et al., 1990; Ewalt and Forbes, 1987).

Moreover, because of the high genetic homology among bacteria of the genus Brucella, there is a demand for the development and validation of highly polymorphic markers to increase sensitivity and resolving power. Recently, Bricker et al.
was extracted using a commercial kit (Puregene, Gentra Systems, Minneapolis, MN). VNTR loci were amplified in independent PCR reactions under the previously described conditions (Bricker et al., 2003). Each 15-μl reaction mixture consisted of 0.6 units of GoTaq® Flexi DNA polymerase (Promega), 1× PCR buffer, 1.5 mM MgCl₂, 0.25 mM dNTP's, 0.2 μM forward and reverse primers and approximately 10 ng of DNA. One of the primer pairs was labelled with Cy5 at the 5' end for detection in an ALFexpress DNA sequencer. The amplicon sizes were calculated from co-migrating size markers in each lane, by the ALFwin Fragment Analyzer (v.1.02 – Amersham Biosciences) and scored by two independent researchers. Some isolates were tested by an independent laboratory (NADC, USDA, Iowa) to confirm and improve the quality of our data. These isolates were also typed with the HOOF-Print protocol, and the amplicon DNAs were sequenced to validate the results.

Typeability and reproducibility were estimated as suggested by the ESGEM (Struelens, 1996). Genetic diversity was quantified by the Simpson's diversity index (Simpson, 1949) and the Hunter–Gaston discrimination index (HGDI; Hunter and Gaston, 1988) via the online tool V-DICE available at the HPA website (http://www.hpa-bioninfotools.org.uk/cgi-bin/DICI/DICI.pl). The discriminatory power of HOOF-Print genotyping was determined for all isolates and for each species. Allelic richness was estimated using rarefaction with the program HP-RARE 1.0 (Kalinowski, 2005). The number of alleles in a sample is a fundamental measure of genetic diversity, however, this diversity measure can be difficult to use because large samples are expected to contain more alleles than small samples. The statistical technique of rarefaction corrects for this sampling disparity (Kalinowski, 2005).

Among the 71 Brucella isolates representing Portuguese diversity (Additional File 1), we found quite good typeability results. Typeability is the ability of getting a measurable and unambiguous result from an experiment. We estimate for all targets tested, a typeability of 98% for B. abortus and 95% for B. melitensis (157/160 and 386/408, respectively). For complete multilocus genotypes, we have a lower typeability, as expected (i.e. 85% for B. abortus and 82% for B. melitensis). The calculated reproducibility of our results (R = 0.983 at the locus level, and R = 0.967 at the composite fingerprint level) was similar to that reported by Bricker and Ewalt (2005) and meets the recommended limit (P ≥ 0.95).

Diversity Indices (HGDI and Simpson's Index) were reasonably high despite our relatively small geographic study area (Table 1). HGDI ranged from 0 to 0.95 when considering all 71 isolates (Table 1). Allelic diversity (e.g. alleles per locus) was comparable to results reported for samples from across the world (Le Fleche et al., 2006; Whatmore et al., 2006) and ranged from 1 to 23 among loci (Table 1). Equivalent results were obtained with Simpson's Index. The Portuguese isolates have more alleles at Locus 1 and 4 than was reported for isolates from across the world (Le Fleche et al., 2006; Whatmore et al., 2006). Allelic richness for both Brucella species was similar (approximately six alleles per locus). These results are highly encouraging and important given the relatively small spatial scale of this study (within Portugal).

The most discriminatory loci were Locus-7 in B. abortus and Locus-1 in B. melitensis with values of 0.94 and 0.90, respectively. The least discriminative locus was Locus-6 for B. abortus (HGDI = 0.56) and Locus-3 for B. melitensis (HGDI = 0). To advance the understanding of the general discriminatory power of each VNTR we compared our diversity indices with those reported by other authors (Additional File 2). Our results are similar to those described by Bricker et al. (2003) and Bricker and Ewalt (2005) but we found a considerably higher diversity at Locus-5 and 8 for B. abortus (e.g. HGDI of 0.912 and 0.680 respectively, compared to 0.04 and 0.0). Most of the diversity at Locus-8 comes from the B.
abortus biovar-3 isolates. This biovar is not found in the USA, which explains the difference between our data and the data originally published by Bricker. Among the Brucella melitensis samples analyzed, VNTR Locus-3 contained a single allele, which is similar to the findings by Bricker et al. (2003). As the genomes of the Brucella species studied have two chromosomes, the probability of non-random associations between loci (GD) is high. Indeed, it is widely accepted that GD between loci can bias phylogenetic and population genetics analysis (Vernez et al., 2005). Thus, we tested our data for GD and found two main linkage groups (i.e., loci with non-random association between pairs of alleles) of five loci in Brucella melitensis (locus 1 + 2 + 4 + 5 + 6 and 4 + 5 + 6 + 7 + 8) and three groups of two loci in Brucella abortus (locus 2 + 8; 3 + 4; and 5 + 6) (*P < 0.05). Therefore, we combined the genotypes into these groups and analyzed data using haplotypes and not individual loci.

An important finding is that the phylogenetic network reconstruction using haplotypes clearly separates biovar 1 and 3 (B. melitensis) when “HOOF-Print” locus-specific genotypes were combined into multilocus haplotypes (Fig. 1). Important also, is to point out that distinction between biovars 1 and 3 of Brucella melitensis was not possible using all loci as independent. It is interesting to note that when applying the same haplotype identification methodology to the available data reported by Whatmore et al. (2006) (Portuguese strains) we obtained similar results (data not shown) suggesting that haplotype identification can improve biovar identification.

In summary, we could discriminate between Brucella melitensis biovars from Portugal only when using haplotype reconstruction, which identifies statistically associated markers as a single multilocus block. This is to our knowledge the first study that tests for improved discrimination power using haplotypes. Identification of haplotypes is widely conducted and recommended in phylogenetics and population genetics and merits further investigation and application in microbial discrimination studies. Finally, the high polymorphism observed in our sample

### Table 1

<table>
<thead>
<tr>
<th>Locus</th>
<th>Brucella spp.</th>
<th>Brucella abortus</th>
<th>Brucella melitensis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ni Na Range (bp)</td>
<td>Diversity Index</td>
<td>Ni Na Range (bp)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HGDI Simpson’s DI</td>
<td></td>
</tr>
<tr>
<td>LOCUS-1</td>
<td>69 23 91–211</td>
<td>0.945 0.931</td>
<td>20 11 92–180</td>
</tr>
<tr>
<td>LOCUS-2</td>
<td>71 5 101–133</td>
<td>0.648 0.639</td>
<td>20 3 101–117</td>
</tr>
<tr>
<td>LOCUS-3</td>
<td>71 5 128–176</td>
<td>0.276 0.273</td>
<td>20 5 128–176</td>
</tr>
<tr>
<td>LOCUS-4</td>
<td>69 20 102–229</td>
<td>0.933 0.919</td>
<td>20 7 102–158</td>
</tr>
<tr>
<td>LOCUS-5</td>
<td>62 14 139–251</td>
<td>0.908 0.893</td>
<td>19 8 139–203</td>
</tr>
<tr>
<td>LOCUS-6</td>
<td>69 9 150–190</td>
<td>0.867 0.854</td>
<td>20 3 159–175</td>
</tr>
<tr>
<td>LOCUS-7</td>
<td>65 12 94–206</td>
<td>0.890 0.877</td>
<td>18 10 102–206</td>
</tr>
<tr>
<td>LOCUS-8</td>
<td>67 8 139–180</td>
<td>0.640 0.631</td>
<td>20 4 140–180</td>
</tr>
<tr>
<td>Total</td>
<td>88 53 63</td>
<td>63</td>
<td></td>
</tr>
</tbody>
</table>

![Fig. 1. Phylogenetic networks of haplotypes from two linkage groups (a and b) showing that biovars 1 and 3 can be distinguished (Brucella melitensis biovar 1, Brucella melitensis biovar 3). Note: Samples number has correspondence with Additional File 1.](image-url)
confirms the usefulness of the HOOF-print loci to discriminate *Brucella* species, biovars, and isolates across a relatively small geographic scale such as Portugal.

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**Appendix A. Supplementary data**


**References**


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