Identification of new IS711 insertion sites in Brucella abortus field isolates

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Abstract

Background: Brucellosis is a zoonosis caused by Brucella spp., a group of highly homogeneous bacteria. The insertion sequence IS711 is characteristic of these bacteria, and occurs in variable numbers and positions, but always constant within a given species. This species-associated polymorphism is used in molecular typing and identification. Field isolates of B. abortus, the most common species infecting cattle, typically carry seven IS711 copies (one truncated). Thus far, IS711 transposition has only been shown in vitro and only for B. ovis and B. pinnipedialis, two species carrying a high number of IS711 copies, but never in other Brucella species, neither in vitro nor in field strains.

Results: We found several B. abortus strains isolated from milk and aborted fetuses that carried additional IS711 copies in two hitherto undescribed insertion sites: one in an intergenic region near to the 3′ end of a putative lactate permease gene and the other interrupting the sequence of a marR transcriptional regulator gene. Interestingly, the second type of insertion was identified in isolates obtained repeatedly from the same herd after successive brucellosis outbreaks, an observation that proves the stability and virulence of the new genotype under natural conditions. Sequence analyses revealed that the new copies probably resulted from the transposition of a single IS711 copy common to all Brucella species sequenced so far.

Conclusions: Our results show that the replicative transposition of IS711 can occur under field conditions. Therefore, it represents an active mechanism for the emergence of genetic diversity in B. abortus thus contributing to intra-species genetic polymorphism.

Background

Brucella is a genus of bacteria causing brucellosis, a zoonosis that affects a large variety of mammals and that is readily transmitted to humans. The genus includes several classical species that can be distinguished by their preferential host range, surface structure, biochemical and physiological features, and genetic markers. This classification is reflected in some degree of genetic polymorphism, one of the main sources of which is the copy number and distribution of IS711 (IS6501) [1,2]. B. melitensis and B. suis contain seven complete IS711 copies [3]. B. abortus carries six complete and one truncated IS711 copies [4], B. ceti and B. pinnipedialis more than 20 copies [5,6] and B. ovis 38 copies [7]. IS711 is very stable: its mobility has been demonstrated only by using a “transposon trap” in vitro in B. ovis and B. pinnipedialis, but not in B. melitensis and B. abortus [3]. Based on this stability, polymorphism at the alkB locus [8] is used to differentiate B. abortus from B. melitensis, B. ovis and B. suis in the AMOS multiplex PCR assay [9].

IS711 stability is not only relevant for Brucella typification: its mobility is implicated in the generation of genetic diversity and speciation, as shown by the distribution of IS711 among the extant Brucella species. Here we report that IS711 transposition and the generation of the associated polymorphism takes place in B. abortus under natural conditions, when genetic drift should be limited by the selective pressure imposed by the host.

Results and discussion

In a previous work with 46 B. abortus strains, it was found that two isolates (B12 and B16) displayed IS711 profiles that were different from that typical of B. abortus.

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field strains [10]. This is confirmed here by the genetic profiling summarized in Table 1, and by the IS711 Southern blot presented in Figure 1. The latter shows that, while the reference strain B. abortus 544 presented seven IS711-carrying fragments, isolates B12 (x-B12), and B16, B49 and B50 (x-B16) displayed an additional one. It is known that RB51, a lipopolysaccharide rough strain obtained from B. abortus 2308 by multiple in vitro passages on antibiotic containing media, harbors eight copies plus an additional one that transposed into the lipopolysaccharide wboA gene [11]. Similarly, B. abortus 2308, a strain isolated more than sixty years ago and extensively replicated in different laboratories carries eight IS711 copies [12,13]. However, the molecular weight of x-B12 and x-B16 fragments (6.6 and 5.5 kb, respectively) was different from those bearing the extra IS711 copies in 2308 (x-08, 1.9 kb that also includes the 3a copy) and RB51 (x-RB51, 1.5 kb) (Figure 1). Interestingly, whereas strain B51, which was isolated from the same sample as B12, displayed the genetic profile typical of B. abortus, strains B16, B49 and B50 showed an identical profile, even though they were from successive outbreaks in the same flock. These results show that it is possible to find B. abortus field isolates with different IS711 distributions.

We characterized the insertion sites in B12 and B16 (and B49 and B50) to ascertain whether they were new or already present in other brucellae. To this end, we carried out IS-anchored PCR using IS711-bound primers plus a decamer of %GC similar to that of the Brucella genome (Table 2). The resulting amplicons ranged from 0.2-3.3 kb (Figure 2A and 2B) with a similar distribution among strains, but with an additional PCR fragment for

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### Table 1 Brucella strains used

| Strain                  | Relevant features                  | Genetic profile by: | Reference |
|-------------------------|-----------------------------------|---------------------|-----------|
| **B. abortus 544**      | Reference strain of biovar 1      | RFLP IS711 Ava-Clai<sup>a</sup> | A         |
| **B. abortus 2308**     | USDA challenge strain; biovar 1   | AMOS enhanced PCR<sup>b</sup> | A         |
| **B. abortus RB51**     | Vaccine rough derivative from 2308|                     | B         |
| **B. abortus B12<sup>c</sup>** | Biovar 1; milk isolate (Río Bueno, Chile; 2004) |                     | C         |
| **B. abortus B16<sup>d</sup>** | Biovar 1; milk isolate (Río Bueno, Chile; 2004) |                     | D         |
| **B. abortus B49<sup>d</sup>** | Biovar 1; aborted fetus isolate (Osorno, Chile; 2002) |                     | E         |
| **B. abortus B50<sup>d</sup>** | Biovar 1; aborted fetus isolate (Osorno, Chile; 2000) |                     | E         |
| **B. abortus B51<sup>c</sup>** | Biovar 1; aborted fetus isolate (Osorno, Chile; 2004) |                     | A         |
| **B. abortus 23/290**   | B. ovis reference strain         |                     | F         |
| **B. ovis 23/290**      | B. ovis type strain               |                     | Np<sup>e</sup> |
| **B. pinnipedialis NCTC 12890** | B. pinnipedialis type strain     |                     | Np<sup>e</sup> |
| **B. abortus 2308 Nal<sup>II</sup>** | Nalidixic acid resistant derivative of 2308 strain |                     | Np<sup>e</sup> |

<sup>a</sup> IS profiles are shown in Figure 1.

<sup>b</sup> A, B. abortus typical pattern; B, B. abortus 2308 pattern; C, B. ovis typical pattern.

<sup>c</sup> B12 and B51 were isolated from the same sample.

<sup>d</sup> B16, B49 and B50 are strains isolated from different outbreaks in the same flock.

<sup>e</sup> Np: Not performed

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**Figure 1** Identification of new IS711 copies in B. abortus B12, B16, B49 and B50 by Southern blot. The new IS711 copies found in field isolates and the additional IS711 present in 2308 and RB51 are indicated on the left. The IS711-nomenclature proposed by Ocampo-Sosa et al. (2008) and the fragment size are indicated on the right (note that x-08 fragment includes both the additional 2308 strain and 3a copies). The signals marked with an * correspond to IS other than IS711 which show cross-hybridization. Capital letters at the bottom indicate the RFLP IS711 Ava-Clai profile (Table 1).
each B12 and B16 strains. Considering that those fragments may contain part of the additional IS copies plus their surrounding sequences, we cloned and sequenced the 3.3 kb and 2.5 kb DNA amplicons of B12 and B16, respectively, and designed flanking primers (Table 2) to confirm the position of the new IS copy. As predicted for the insertion of complete IS\textsuperscript{711} copies of 842 bp in length, specific PCR products of 1077 bp (B12) and 1142 bp (B16) were amplified (Figure 2C and 2D). We believe that an IS replicative transposition is the most plausible explanation for these results. In fact, the sequence analysis suggested that transposition had occurred by a canonical TA duplication at YTAR site (R, purine; Y, pyrimidine). In strain B12, this site was in an intergenic region between a lactate permease gene (lldP) and BruAb1\textsubscript{0736} (hypothetical protein) (Figure 3, upper panel) corresponding to a 103 bp Bru-RS1 element, a palindromic repeat sequence that represents a putative insertion site for IS\textsuperscript{711} [14]. In contrast, the IS\textsuperscript{711} extra copy in B16, B49 and B50 was interrupting an ORF encoding a transcriptional regulator of the MarR family (BruAb2\textsubscript{0461}, Figure 3 lower panel). Similarity searches showed that the B12 and B16 sites did not match with any of the IS\textsuperscript{711} loci previously reported for \textit{B. abortus} or even with the novel IS\textsuperscript{711} sites recently described for \textit{Brucella} marine mammal strains [6], although the B16 site was found in \textit{B. ovis}. To confirm these findings and to investigate whether these

### Table 2 Primers used in this work

| Name          | Sequence (5’-3’)                                      | Reference |
|---------------|------------------------------------------------------|-----------|
| 711d          | CATATGATGGGACCAAACACCTAGGG [19]                      |           |
| 711u          | CACAAGACCTGCGTTGCAGAG [19]                           |           |
| RB51          | CCCCGGAAGATAGGTGCTGCC [12]                           |           |
| IS711out      | CAAGTGGAAAGCTATGTCGC                                 | This work |
| P5            | CGGCCCGGGT [20]                                      |           |
| BruAb1\textsubscript{0736}F | TTGGTTCTCTTGCGAGACAT                                  | This work |
| BruAb1\textsubscript{0737}R | AACCTGCGTTATGTCACA                                     | This work |
| BruAb2\textsubscript{0461}F | ATCGGCTTTCTGCGGAATC                                   | This work |
| BruAb2\textsubscript{0461}R | TCGTTGCGATCTGCGAG                                     | This work |
| marR\textsuperscript{F1} | GACGTTGGAGAAGAAACCTA                                  | This work |
| marR\textsuperscript{R2} | ACTCGGCAAAACCTGATAA                                   | This work |
| marR\textsuperscript{F3} | TTATCAGGGTTTGGCCGGAGTCACATTGGAGAAGTCATG            | This work |
| marR\textsuperscript{R4} | CGCTCTGGGTACGCTATT                                 | This work |

![Image](http://www.biomedcentral.com/1471-2180/11/176)
sites were also present in the genomes (not available in databases) of the Brucella species carrying a high-copy number of IS711, we carried out PCR assays with B. ovis, B. ceti and B. pinnipedialis DNAs. For the B12-specific IS711, PCR amplifications with flanking primers yielded an IS-empty locus fragment (not shown). In contrast, the PCR amplifying the B16 fragment yielded the predicted 1142 bp fragment in B. ovis but not in B. ceti or B. pinnipedialis (Additional file 1).

The x-B12 and x-B16 IS711 sequences were nearly identical to that of IS711_1a and depicted only changes in a few nucleotides (Figure 4A). On the basis of the high IS711 sequence similarity across sequenced B. abortus strains, we performed a cluster analysis between IS711 loci found in B. abortus 9-941 and those additional ones found in 2308, RB51, B12 and B16 strains to get insight about their origin (Figure 4B). Although as expected, the analysis disclosed only low sequence dissimilarity, it suggested that the new copies might derive from IS711_1a. Since a previous work has shown that the IS711_xa in the B. abortus alkB locus and the IS711_x-08 in strain 2308 are identical to IS711_1a [3], the inclusion of IS711_x-B12 and IS711_x-B16 in the same cluster supports the hypothesis that IS711_1a is more active than other copies in the B. abortus genome and can transpose into new sites or even into sites shared with related species.

IS transposition can disrupt genes and produce negative polar effects, but also cause beneficial changes by remodeling genomes through long range recombination [15]. In the case of strain B12, it is uncertain whether the intergenic position of IS711 disturbs the expression of nearby genes. Most IS711 studied in detail (1a, 2a, 3a, 5a, 6a, xa and x-08) are also located within intergenic regions showing that transposition is mostly viable when occurring into neutral sites. However, the extra IS711 copy in B16, B49 and B50 interrupts a putative transcriptional regulator that is expressed during the late-logarithmic phase of growth in B. melitensis (BMEII0520) [16] and, interestingly, these strains did not show urease activity, a factor that has been proposed to favor Brucella gastrointestinal infections in mice [17]. We investigated whether the marR mutation was involved in the urease-negative phenotype by constructing a B. abortus 2308 ΔmarR mutant. This mutant displayed urease activity (not shown), suggesting that the absence of urease in B16, B49 and B50 is probably caused by mutation(s) in ure genes [17]. The fact that these urease negative marR mutant strains were repeatedly isolated from aborted fetuses for at least four years questions the relevance of this factor in placental colonization and abortion induction. Research is in progress to characterize the genetic background of this urease negative phenotype.

**Conclusions**

In this report, we have provided evidence that IS711 polymorphism occurs in B. abortus field strains. The fact that such polymorphism can take place in sites shared with related species points out the relevance of a multiple-marker approach in molecular typing of Brucella species. In addition, our results suggest that the extra IS copies might originate from what seems to be the most active IS711 copy. Although the environmental
signals involved in the activation of the transposase remain unknown, host-pathogen interactions may play a role. Further work is needed to elucidate if changes promoted by IS transposition are associated with virulence fluctuations in this pathogen.

Methods
Bacterial strains, growth conditions, plasmids and DNA manipulation
The Brucella strains studied are listed in Table 1 and the E. coli strains and plasmids used are in the Additional file 2. Bacteria were stored in tryptic soy broth (Becton Dickinson, Sparks, Md) with 20% glycerol at -70°C and, for routine use, grown on tryptic soy agar (Becton Dickinson, Sparks, Md) with 20% glycerol at 37°C. Plasmids were obtained with Qiaprep (Qiagen, Hilden, Germany). PCR products and genomic DNA were purified with a QiaexII kit (Qiagen) or by standard protocols [18].

Molecular typing techniques
AMOS PCR was carried out as described before [12]. IS711 Southern blots, genomic DNA (1-2 μg) was digested with Aval and Clal (Fermentas Inc, Burlington, Canada) at 37°C overnight, the fragments resolved in 1.0% agarose at 15 mA for 10 h, blotted on nylon, fixed at 80°C for 30 min and probed with a biotin-labelled IS711 fragment obtained by PCR with primers 711u and 711d (Table 2). Hybridization was performed at 42°C for 2 h, and detected by chemiluminescence (KPL, Gaithersburg, MD) [19].

Genome mapping of new IS711 insertion sites
For IS-anchored PCR, we adapted a protocol previously described [20]. IS711-bound primers RB51 and IS711out in combination with an arbitrary primer P5 (Table 2) were used to generate a pattern of PCR products specific for diverse IS positions. The reaction mixture contained 0.2 μM of RB51 or IS711out primers and P5 decamer, 5.0 μl of 10X enzyme buffer, 2 mM of MgCl2, 0.4 mM of dNTP, 1 U of Taq polymerase (Invitrogen) and 10 ng of genomic DNA. The amplification conditions were: 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 30 sec; annealing at 55°C for 30 sec; extension at 72°C for 2 min; final extension at 72°C for 5 min. Amplicons were electrophoresed in 1.5% agarose in 20 mM Tris, 20 mM acetic acid, 1 mM EDTA, and detected with ethidium bromide.

Cloning and sequence analysis
Specific IS-anchored and flanking PCR products purified from gels were cloned into the pCR2.1 vector (Invitrogen) and sequenced by fluorescence-labeled dideoxynucleotide technology (Macrogen Inc, Seoul, South Korea). Sequences were analyzed by BLASTN (http://www.ncbi.nlm.nih.gov/). Comparison of the IS711 sequences in the B. abortus 9-941 genome (accession numbers AE017223 and AE017224) [4] and the new IS711 was performed with ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2). Sequences of new IS711 were deposited under GenBank accession numbers: JF345125 and JF345126.

Construction of B. abortus 2308 ΔmarR mutant
A B. abortus 2308 NalR ΔmarR non polar mutant was constructed by allelic exchange [21] with primers designed on the sequence of marR (BAB2_0468, the marR homologous). Briefly, two fragments generated with primer pairs marR-F1, R2 and marR-F3, R4 (Table 2) were ligated by overlapping PCR and the resulting fragment (containing a ΔmarR lacking the nucleotides corresponding to amino acids 13-120) was cloned into pCR2.1 to produce plasmid pMM19 (Additional file 2). The BamHI-NotI fragment of pMM19 was subcloned into plasmid pQK [22] to generate the pMM21 suicide vector (Additional file 2), which was transferred to B. abortus 2308 NalR by conjugation with a suitable E. coli strain [23]. Nalidixic acid and sucrose resistant clones were screened by PCR, and tested for urease [17].

Additional material
Additional file 1: PCR analysis for the presence of x-B16 fragment in B. ovis, B. ceti and B. pinnipedialis. Additional file 1 is a word file displaying a picture of PCR results.
Additional file 2: E. coli strains and plasmids. Additional file 2 is a word file displaying a table with E. coli strains and plasmids used in this work.

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Authors’ contributions
MM conceived the study, participated in its design, accomplished computational analysis, and carried out molecular typing, mutagenesis and PCR assays. MU performed PCR assays and cloning procedures. ILG provided financial support and helped to draft the manuscript. IM and MW wrote the manuscript. AMZ participated in the design, coordination and financial support of the study and helped to draft the manuscript. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.
