Genetic Homogeneity Of Clinical Isolates Of Brucella Melitensis: A Single Ribotype

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Abstract

Introduction: One of the genetic targets frequently used for strain identification and strain phylogeny is the rRNA operon, particularly the 16S rRNA gene. Ribosomal genes in Brucella have been implicated in recombination events that promoted the division of a chromosome into two chromosomes. These genes are highly conserved and diverge very slowly.

Methods: Brucella was cultured or isolated from 100 blood samples that were collected from clinical cases from a rural endemic area in Jordan. The primers used were genus-specific designed to amplify a 905 bp fragment of the highly conserved region within the 16S rRNA of the genus Brucella. The PCR products were subjected to restriction fragment length polymorphism analysis by digestion with HaeIII and MspI separately.

Results: All isolates had the same rRNA gene restriction pattern and identical ribotype. Ribotyping confirms the high levels of DNA-DNA relatedness among Brucella species supporting the conclusion that Brucella is actually a monospecific genus.

Conclusions: Results demonstrated the limited genetic diversity of Brucella isolates and that the rRNA gene might not be a good locus to differentiate between Brucella strains.

Introduction

Brucellosis is a worldwide zoonosis that is also prevalent in the northeast rural regions of Jordan [1], where Brucella melitensis was isolated from human cases and aborted animals in Jordan [2,3,4]. According to the World Health Organization, half a million new cases are reported each year worldwide [5]. These numbers greatly underestimate the true incidence of human disease because the clinical picture of human brucellosis is extremely variable [6]. Six species (B. abortus, B. melitensis, B. suis, B. ovis, B. canis, and B. neotomae) are recognized; and it has been proposed that B. melitensis be the genomic species [7]. On the basis of the 16S rRNA sequence comparisons, the genus Brucella has been found to be a member of the alpha-2 subdivision of the class Proteobacteria, closely related to Rochalimaea, Rhizobium, and Agrobacterium spp. [8,9].

Numerous PCR-based assays have been developed for the identification of Brucella to improve diagnostic capabilities [10,11] during both primary infections and relapses of the disease [12]. Moreover, molecular probes have been developed for typing Brucella strains [13,14]. Collectively, the repertoire of assays addresses several aspects of the diagnostic process. For some purposes, the simple identification of Brucella is adequate (e.g. diagnosis of human brucellosis or contamination of food products). In these cases, a genus-specific PCR assay is sufficient; these assays tend to be simple, robust, and somewhat permissive of environmental influences. The main genetic targets utilized for these applications are the Brucella BCSP31 gene and the 16S-23S rRNA operon. Other instances require identification of the Brucella species involved. Despite the high degree of DNA homology within the genus Brucella, several methods, including a PCR-restriction fragment length polymorphism (PCR-RFLP) and southern blot, have been developed, which allow to a certain extent the differentiation between Brucella species and some of their biovars. DNA heterogeneity among members of the genus Brucella was demonstrated with the arbitrarily primed PCR (AP-PCR), where Brucella strains can be distinguished according to the banding patterns of their amplified DNA [15].

Genetic relatedness within Brucella has been based on a comparison of omp2 sequences [16] using a combination of omp-31 PCR-RFLP patterns and southern blot hybridization profiles. Brucella species were differentiated with the sole exception of Brucella neotomae. DNA restriction maps obtained from various species [17], and multilocus enzyme electrophoresis [18] has been frequently used for the same purpose.

One of the genetic targets frequently used for strain identification and strain phylogeny is the rRNA operon, particularly the 16S rRNA gene, a region for which extensive base pair sequence information exists for many microorganisms, including Brucella species. Ribosomal genes in Brucella have been implicated in recombination events that promoted the division of a
chromosome into two chromosomes [19].

The objective of the present study was to assess the genetic diversity among *Brucella melitensis* isolates by typing a large number that were isolated between 1999 and 2001 from blood specimens of brucellosis patients residing in a rural endemic area in northeast Jordan.

A ribotyping method was used for the characterization of individual isolates of *Brucella*. This technique combines PCR amplification of a portion of the 16S rRNA gene with restriction analysis of the amplified product.

**Methods**

All subjects included in the study consent to the collection of the specimen and the University review committee approved the study.

One hundred blood specimens were collected from outpatients clinically diagnosed with brucellosis as previously described [1]. These patients were seen in health centers in the Jordan Badia, a rural area in the northeast of Jordan known to be endemic for brucellosis.

**Bacterial strains and growth conditions.** Twenty out of the 100 *Brucella* strains used in this study were grown on tryptic soy agar at 37°C for 48 h. All *Brucella* single-colony isolates were tested for their Gram reaction and were identified by standard methods and PCR assay.

**Extraction of genomic DNA from blood specimens.** DNA was extracted from the blood specimens using a commercial purification system (Wizard Genomic DNA Purification Kit, Promega, Madison, WI) according to the manufacturer’s instructions for DNA purification from blood. Final pellets were resuspended in 50 mL of TE (10 mM Tris, 1 mM EDTA, pH 7.2).

**DNA amplification by PCR.** The *Brucella* DNA-Detect PCR Kit (Vita-Tech International Inc., CAN), including the reagents and oligonucleotid primers designed to amplify all species of *Brucella*, was used for the detection of *Brucella* spp. in the samples. The F4-R2 primers used in the kit are genus-specific primers derived from highly conserved regions correspond to positions 63–79, and 947–966 of the *B. abortus* 16S rRNA gene that are designed to amplify 905-bp fragment [20].

**Amplification conditions.** The reaction mixture contained 5 :L of 10x PCR buffer, 1 :L of the primer mix, 2 :L of dNTP mix (5 mM each), 3 :L of 25 mM MgCl2, 1 unit of Taq DNA polymerase, 5 :L of sample DNA in a volume of 50 :L. The same mixture was used with the positive control provided by the kit. Negative controls were routinely processed to monitor contamination with *Brucella* DNA, they included distilled water alone, or the reaction mixture containing all of the reagents, but lacking template DNA. All standard precautions recommended for prevention of contamination with DNA and amplicons were undertaken.

The reaction was performed in a thermal cycler (GeneAmp PCR System 9700, Perkin Elmer, Norwalk, Conn.). The cycling conditions were an initial denaturation at 95°C for 5 min, template denaturation at 95°C for 30s, annealing at 54°C for 90s, and primer extension at 72°C for 90s for a total of 35 cycles, with a final extension at 72°C for 6 min. A sample was considered positive when the size of the DNA band (905 bp) matched with that of the positive control.

Twelve micro liters of the PCR product were run by electrophoresis in a 2% agarose gel stained with ethidium bromide, and amplification products were detected by visualization of the bands under UV light.

**Restriction enzyme analysis.** In preliminary experiments, PCR products of 25 positive samples were subjected to digestion with three restriction enzymes *HaeIII*, *MspI*, and *TaqI* (Promega, Madison, Wis.). All digestions were performed under conditions defined by the manufacturer.

No digestion of any of the samples was observed with *TaqI*. Based on these results, *HaeIII*, and *MspI* were chosen for the subsequent studies.

The amplified fragments of all samples were subjected to RFLP analysis by digestion with *HaeIII* and *MspI* separately, in a total volume of 20 :L and resolution of the resultant digestion products was achieved by electrophoresis on a 2.8% agarose gel containing 0.5 :g/ml of ethidium bromide. The digestion reaction was repeated twice for each PCR product.

**Results**

The restriction enzyme analysis for the 100 isolates tested revealed a single restriction pattern for each enzyme (*HaeIII*, and *MspI*). The *HaeIII* restriction profile contained three fragments of 450, 400 and 55 bp. The *MspI* restriction profile contained three fragments of 750, 100, and 50 bp. No variation from these patterns was observed for any of the isolates.

The 16S rRNA gene known to be highly conserved among bacteria diverge very slowly and is frequently used in the determination of organism relatedness [21]. The DNA sequences from separate species within a
genus will differ by only a few percent, and sequence identity among the 16S rRNA sequences is typically interpreted as indicating a single species [22]. However, variable regions do exist within the gene, and some sequences within these variable regions are unique to certain species. Minor base substitutions can result in changes in restriction endonuclease recognition sites and consequently in variant RFLP patterns for members of the same species.

Consensus does not yet exist regarding how similar the 16S rRNA sequences of two organisms must be in order for them to be considered to belong to the same species [23]. In a study of the phylogenetic relatedness of Bacillus species [24], stated that for sequences of approximately 1,000 bases, 16S rRNA sequences from most species that are recognized to be separate differ in at least 1.5% of their bases (98.5% similarity). Isolates belonging to the same species generally have a similarity of at least 99.2%. Thus, it seems reasonable to consider Brucella isolates to belong to different species if they have # 98.5% similarity.

A number of studies reported on the application of PCR-RFLP analysis for typing of Brucella and other bacteria such as Borrelia burgdorferi sensu lato [25], and Nocardia isolates [23] from varied biological and geographic sources. This method revealed a relationship between DNA fingerprints, species, and pathovars, which could shed light on problems concerning the classification and evolution of members of the genus Brucella. A major advantage of this approach is its applicability to uncultured specimens; it is a rapid and direct method for genotyping of Brucella clinical isolates. Studies correlating the genotypes of cultured patient isolates with those obtained by direct PCR-RFLP analysis of animal isolates are limited.

The present study has focused on the use of this method in the evaluation of a large number of clinical isolates. The same gels, buffers, ethidium bromide concentration, and electrophoresis conditions were used all through the experiments to avoid differences from predicted band sizes due to variations in these concentrations. Agarose concentration and electrophoresis conditions were selected to allow distinction of as many different band sizes as possible. All the isolates identified as B. melitensis were obtained from patients’ blood samples from the same endemic area, and the strains tested are supposed to be representatives of strains present in that area. The results showed that they all have the same rRNA gene RFLP pattern and one identical ribotype. These results are in agreement with a previous study of 99 Brucella isolates by the multilocus enzyme electrophoresis method [18] using the restriction enzymes MspI, HinfI, HhaI, and Sau3AI, which showed that all isolates had the same rRNA gene RFLP patterns. These results reveal a high conservation of the restriction site or gene order [26], and are in agreement with what was previously reported [17] on the similarity of the restriction maps of B. melitensis and other six Brucella spp.

The findings of the current study indicate that strains within a biovar have exactly the same restriction pattern, which has been reported by others [27]. Brucella species were reported to exhibit different XbaI restriction patterns, and biovars within a species were less polymorphic [28]. Minor profile differences allow the discrimination between most biovars within a species. However, the differences in the DNA patterns of different field strains of biovar 2 of B. melitensis were not sufficient to serve as markers for epidemiological studies [27].

The same was concluded by a study of seven biosynthetic genes of the WB locus of Brucella melitensis 16M involved in the biosynthesis of the lipopolysaccharide O-side chain, which have been recently identified [29], and analyzed by PCR-RFLP for polymorphism among Brucella spp. Results showed that the seven genes are highly conserved and occur even in the naturally rough species B. ovis and B. canis, and also in rough strains of B. abortus and B. melitensis. Nevertheless, the few polymorphisms that were observed consisted of absence of additional restriction sites sometimes allowing differentiation at the species level (e.g. B. ovis ), the biovar or strain level. There were no apparent deletions or insertions in the PCR-amplified genes in any of the Brucella strains studied, and was concluded that these genes are highly conserved among Brucella spp. and may have been acquired before species differentiation.

The high levels of DNA-DNA relatedness among Brucella species shown by hybridization and other genetic analyses led to the conclusion that Brucella is actually a monospecific genus [7]. The limited genetic diversity of Brucella spp. might be due to their fastidious growth requirements, and therefore cannot actively multiply in the environment, but only in infected host. Moreover, since mechanism of genetic exchange, such as plasmid, temperate bacteriophage, or transformation, have never been demonstrated to occur naturally in Brucella [17], each species is genetically isolated, despite the frequent coexistence of different strains infecting different animals in the same farm. Although, B. melitensis, and B. abortus are
pathogenic to humans, their virulence is restricted to certain hosts since B. melitensis infects goats and sheep, while B. abortus, infects cattle. The restricted host range exerts specific selective constraints, conductive to the selection of a particular clone in pathogens known to be host specific such as Salmonella typhi [17]. Pathogens, especially those grow intracellularly, have a narrow diversity, which may reflect their habitat constraints [30]. Thus, limited genetic diversity has been also obtained with Yersinia ruckeri, and Salmonella enterica serovar Paratyphi B.

Conclusions

The results demonstrate the genetic homogeneity of Brucella isolates and suggest that the rRNA gene might not be a good locus to differentiate between Brucella strains.

Authors Contribution(s)

Prof. Laila Nimri: experimental design, execution of experiments, data analysis, writing the manuscript.

Dr. Raymond Batchoun: execution of experiments, helped in writing the manuscript

References

1. Nimri LF. Diagnosis of relapsed cases and post treatment follow-up of human brucellosis by PCR assay. BMC Infect Dis 2003;3:5.
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