Using of VNTR method (MLVA16) for investigation of linking between human and animal Brucella isolates in Iran

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Abstract

Background

Epidemiological studies are important tools to assess the diversity among Brucella strains and to estimate the epidemiological relationship of isolates from different geographical origin. For this reason purpose of this study was applied the MLVA16 (A multi locus variable number of tandem repeat analysis) to investigate and to determine the diversity among Brucella strains for epidemiological purposes in human and animal and determine the most common genotypes among Brucella strains in Iran. After isolation and identification of 54 strains of Human and animals Brucella from three regions of Iran, DNA genomic extracted and Multiple-Locus variable number tandem repeat with 3 pannel used for genotyping of isolates. Analysis of PCR product size was carried and converted to repeat unit numbers using a published allele numbering system and as a character data a set were imported into Bionumerics.

Results

Three of isolates (5.55%) were Brucella abortus and fifty one (94.44%) were Brucella melitensis. Using MLVA16 genotyping 54 isolates with 80% of genetic similarity coefficient divided into 46 genotypes that 22 genotypes were represented by a single isolates, 4 by 2 isolates, 2 by three isolates, 1 by 4 isolates and 2 by 7 isolates. The most prevalent genotype was revealed in 14 strains. There were two more frequent genotypes that each contains 7 isolates which one of them was restricted to one geographic region. Discriminatory power for each locus determined in this study and displayed that panel 2B have a high polymorphism with a discretionary power for Bruce04 (0.837), Bruce30 (0.806), Bruce 09 (0.787), Bruce 07 (0.772), Bruce16 (0.766).

Conclusion

The MLVA16 analysis on 54 Brucella isolates showed a high polymorphic of genotypes. Only two groups contain 7 isolates related together and only one of this groups belonging to two separated regions.

Background

Brucellosis is a zoonotic disease caused by the bacteria of genus *Brucella* and transmitted through direct or indirect contact to animal [1]. Brucellae based on differences in pathogenicity, phenotypic characteristics and host reference were classified within eleven species [2, 3]. Four of species including *Brucella abortus, Brucella melitensis, Brucella canis and Brucella suis* are known to infect human. Enormous economic losses and public health problems occur due to abortion, infertility in livestock, weak offspring, decreased milk production and morbidity in human. Although Brucellosis has been eradicated in the USA, Canada, North Europe, Australia, this disease is highly prevalent in central Asia, the Middle East, the Mediterranean region, Africa and Latin America [4]. Iran is an endemic area for Brucellosis and there is a main risk of Brucella transmission from eastern and western neighbors such as Iraq, Pakistan and Afghanistan due to don't have high quality veterinary services for controlling animal disease [5, 6].

Despite, 500000 Brucellosis human cases reported around the world in every year, there are the numbers of undetected and neglected cases [7]. The key control for Brucellosis in human is control of this disease in animal and using of epidemiological studies to assess the diversity among strains for epidemiological purpose in human brucellosis and to estimate the epidemiological relationship of isolates from different geographical origin [8, 9].

The classical phenotyping methods such as serotyping, phage typing, metabolic profiles and sensitivity to dyes have been employed to subtype Brucellosis, but these techniques are available in reference laboratories only, have a limited discriminatory power, time consuming, require manipulating the living agent and in a lake of standardization interpretation of this method cause difficulties [10].

For these limited in phenotyping methods, bacterial typing shift towards molecular identification which investigate epidemiological relationships among isolates and source of infection. Several molecular typing methods have been introduced such as random amplified polymorphic DNA (RAPD)-PCR, amplified fragment-length polymorphism (AFLP), Pulse field gel electrophoresis (PFGE), Polymerase chain reaction restriction fragment length polymorphism, Multilocus sequence typing (MLST) and Multiple Loci VNTR (Variable number tandem repeats) analysis (MLVA). MLVA method introduced as an effective and rapid tool which monitors the variability in the copy numbers of tandem repeat units (TRS) with higher discriminatory power [11]. This method not only uses in outbreak and epidemiological trace-back investigations but also use in confirmatory laboratory or food borne acquired infections. TR sequences are multiple allels can be presented at a single locus, and based on their size differences can be easily resolved through agarose electrophoresis or capillary electrophoresis equipment [12]. MLVA has been proven to be a good technique for the assessment of pathogenic bacteria such as *Brucella* that display very little genomic diversity. MLVA schemes with 21, 15 and 16 loci (MLVA21, 15 and 16) have been published. MLVA 16 has been proposed by Al-Dahank et al with eight minisatellite markers (panel1: Bruce06, Bruce 08, Bruce11, Bruce 12, Bruce 42, Bruce 43, Bruce 45 and Bruce 55) for species identification and eight microsatellite markers (panel2A: Bruce18, Bruce 19, Bruce 21 and 2B: Bruce 04, Bruce 07, Bruce 09, Bruce 16 and Bruce 30) for determine the most common genotypes and the further subspecies differentiation [13]. The genetic diversity of *Brucella* strains isolated from human and animal infection has not yet been investigated in Iran. The main objectives of this study was applied the MLVA16 assay to investigate and to determine the diversity among *Brucella* strains for epidemiological purposes in human and animal and determine the most common genotypes among *Brucella* strains in Iran.

Results

Characteristics of patients and Brucella isolates

The 54 Brucella isolates were respectively isolated from blood (62.96%) and CSf (3.70%) from human and blood (16.66%), spleen (5.55%) and liver (11.11%) from animals. Human patients were 26 male (48.14%) and 10 female (18.51%) and animal patient including 15 sheep (27.77%) and 3 (5.55%) cattle). Isolates were collected from three states including Hamedan (59.25%), Arak (7.40%) and Tehran (33.33%)

of Iran. All of isolates were identified as Brucella melitensis and Brucella abortus. Three of isolates (5.55%) were Brucella abortus and fifty one (94.44%) were Brucella melitensis. The mean age of the 36 patients was 45 (range 6-80 years) and ratio of male to female was 2.6.

MLVA16 genotyping results

The complete MLVA16 assay including panel 1, 2A and 2B loci performed. The random repeat unit of 16 Loci range from 9 bp greater. The PCR products for 16 loci were converted to TRs copy number. Basis on published data, the polymorphism of each of 16 VNTR loci was analyzed in the MLVA-16 method. Using MLVA16 genotyping 54 isolates with 80% of genetic similarity coefficient divided into 46 genotypes that 22 genotypes were represented by a single isolates, 4 by 2 isolates, 2 by three isolates, 1 by 4 isolates and 2 by 7 isolates (figure 1). The most prevalent genotype was revealed in 14 strains. There were two more frequent genotypes that each contains 7 isolates which one of them was restricted to one geographic region (Hamadan), whereas other most prevalent genotype was present in two regions (Hamadan and Tehran). Genotypes with two or three isolates were present in Tehran and Hamadan provinces (figure 2). Discriminatory power for each locus determined in this study and displayed that panel 2B have a high polymorphism with a discretionary power for Bruce04 (0.837), Bruce30 (0.806), Bruce 09 (0.787), Bruce 07 (0.772), Bruce16 (0.766). Panel 2A displayed a moderate variability which discriminatory power for Bruce 18(0.805), Bruce 19(0.568), and Bruce 21(0.358). Panel 1 displays a discriminatory power in Bruce06 (0.204), Bruce 08(0.654), Bruce 11(0.673), Bruce 12(0.598), Bruce 42(0.408), Bruce 43(0.541), Bruce 45(0.713), Bruce 55(0.730) respectively.

Discussion

In the current study a total of 54 strains of Brucella were collected from human and animal patients and then MLVA16 used to assess the genetic diversity among strains of Brucella from three regions of Iran. 36 isolates (66.66%) were from humans and 18 (33.33%) isolates from animals. Three of isolates (5.55%) were identified Brucella abortus and the rest were Brucella melitensis. Brucellosis is an atypical zoonotic disease and constant circulation monitoring in animal will contribute to the control of human brucellosis [14, 15]. Human brucellosis is most often linked with animal husbandry or consuming unpasteurized milk [16-18]. MLVA16 is a proper typing for epidemiological relationship of strains in an outbreak or connected outbreak to avoid excessive classical epidemiological investigation [19, 20]. MLVA16 with 80% of genetic similarity coefficient yielded total 46 genotypes that 22 genotypes were singleton. The frequency of different MLVA genotyps varied among the three regions. Three isolates of B.melitensis that collected from two of sheep (blood and spleen), one of cattle (liver) and one of human (CSF) in Arak were as genotyping distinct. Two Brucella abortus strains isolated from Blood of human and one of them from blood of cattle. The results obtained show no correlation between genotypes of Brucella abortus in cattle and genotypes observed in both of male.

Fifty B. melitensis isolates from blood of human patients in Hamadan shared the same genotype as B. melitensis from blood of sheep and B. abortus isolates from spleen of cattle in Hamadan, suggest a

possible epidemiological connection. Four other genotypes that isolated from blood of human patients in Hamedan were very closely related and differ by single repeat unit differences at one or two of the most variant loci. Variety in single or double locus may reflect the microevolution of shared B.melitensis strains. Additionally three B. melitensis strains isolated from blood of three human patients in Hamadan and Tehran, two B. melitensis isolated from blood of strains, two strains isolated from blood of one human patient and one B. melitensis strain from liver of sheep have a complete matching. These results suggest that transmission from animal to human may occur. Because control measurement such as financial compensation of owners of slaughtered seropositive cattle and no such measure exists for sheep or goat, so sheep infected with Brucella are one of the main sources for human and animal brucellosis in Iran. Similar to previous studies, the highest Diversity discriminatory power were also found for panel 2B loci (0.7936) than those of panel 1 (0.4760) and 2A (0.557). These results revealed that the loci of panel 1 are more conserved than those in panel 2 panel 1 including minisatellite loci with repeat unit length above 9bp versus of panel 2 with more heterogeneity and microsatellite loci. The highest diversity discriminatory power related to Bruce30 (0.806) of panel IIB followed by Bruce18 (0.805) of panel IIA.

Conclusion

The MLVA16 analysis on 54 Brucella isolates showed a high polymorphic of genotypes. Only two groups contain 7 isolates related together and only one of this groups belonging to two separated regions.

Methods

Isolation of Brucella SPP and species determination

The clinical samples collected from human and animals suffered from brucellosis. Culture isolation of Brucella SPP was achieved by streaking the agar supplemented with 10% horse serum. The inoculated plates were incubated at 37 C aerobically for 3-5 days. Single colonies were analyzed microscopically using the Gram stain and biochemical (urease, oxidase and catalase) and motility tests. Colonies confirmed as Brucella SPP were submitted to species identification by PCR methods.

Preparation of Brucella DNA and amplification of Loci

Brucella DNA was extracted using the PCR template preparation Kit (Roche Diagnostics, Germany). MLVA genotyping was performed as previously described by Flech et al and completed by Al Dahouk et al. This method including of 3 panels named pannel1, panel 2A and panel 2B. The 16 primer pairs were divided into three groups. Pannel1 consists of eight minisatellite loci (Broce06, 08, 11, 12, 43, 45, 55), panel 2A (Bruce18, 19, 21) and panel 2B (Bruce04, 07, 09, 16 and 30). PCR amplification was performed in a total volume of 15 µl containing 1ng of DNA, 1X PCR reaction buffer, 1U of Taq DNA polymerase, 200 Mm of each deoxynucleotide triphosphate, and 0.3 Mm of each flanking primers. Amplification was performed in an Ependrof termocycler. An initial denaturation step at 96C for 5 minutes was followed by 30 cycles of

denaturation at 96C for 30S, annealing at 60C for 30s, and elongation at 70C for 1min. The final extension step was performed at 70 for 5 min. Five microliters of amplification product were loaded on 2.5% agarose gel and run under a voltage of 8 V/cm. Gel image were recorded.

Analysis of MLVA data

Analysis of PCR product size was carried and converted to repeat unit numbers using a published allele numbering system and as a character data a set were imported into Bionumerics. For quantification of polymorphism at each locus, Hunted and Gaston diversity index (HGDI), available on the HPA website were used. Categorical coefficient and unweighted pair group methods were applied to clustering analysis. Minimum spanning trees were constructed.

Abbreviations

RAPD-PCR
random amplified polymorphic DNA
AFLP
amplified fragment-length polymorphism
PFGE
Pulse field gel electrophoresis
MLST
Multilocus sequence typing
VNTR
Variable number tandem repeats
MLVA
Multi locus variable number of tandem repeat analysis
TRS
tandem repeat units

Declarations

Availability of data and materials

Data are available upon request from corresponding author.

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Not applicable.

Authors' Contributions

SM initiated and supervised the study. SM and FM designed the experiments. FM and AN conducted the experiments. AN conducted the analysis. SM, FM and AN has written the manuscript. All authors have

read and approved the manuscript.

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Ethics approval and consent to participate

This study was carried according to approved protocol by Ethics committees of Iran University of medical sciences.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

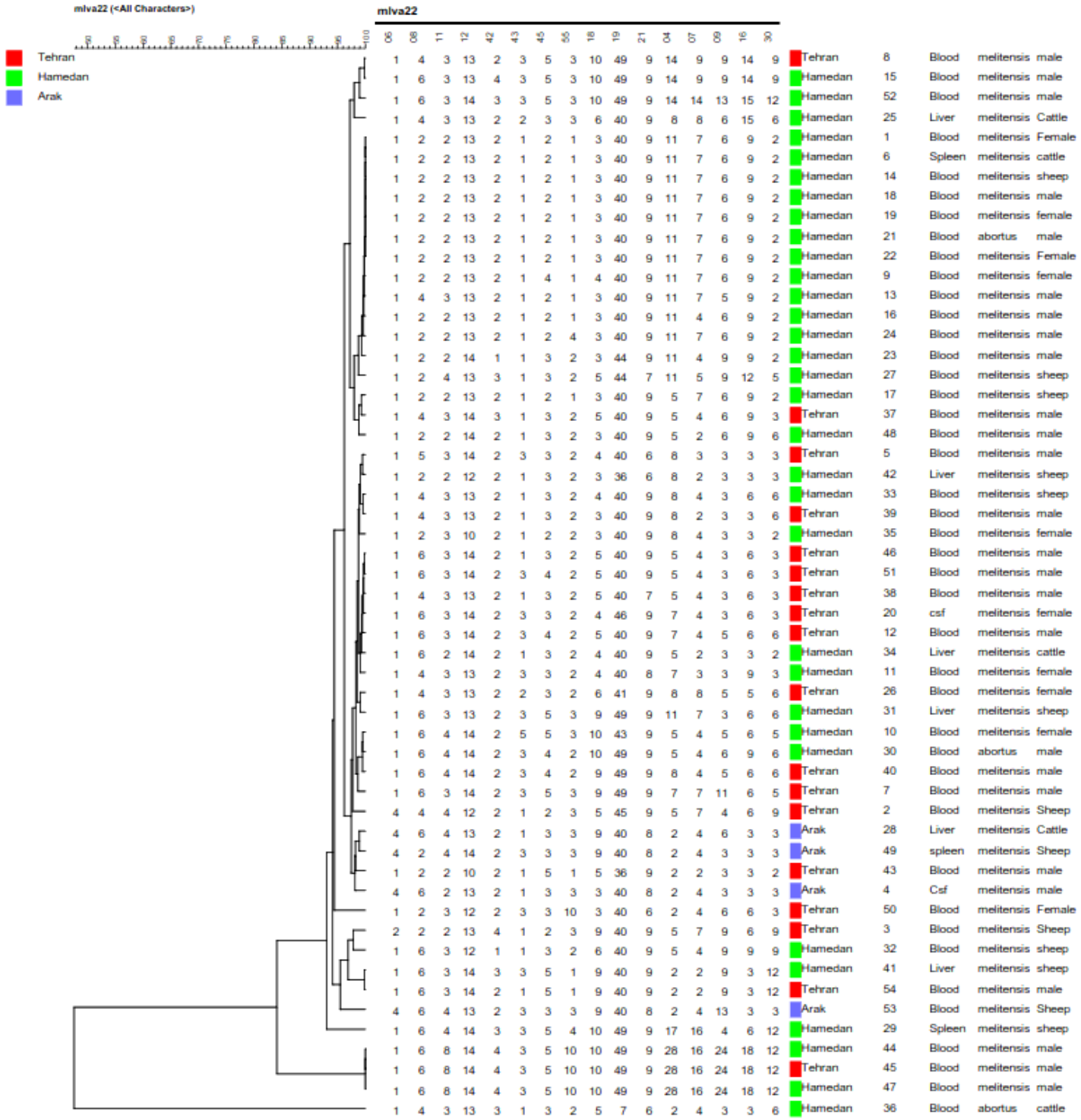

Figure 1

Dendrogram based on MLVA 16 assays on 54 isolates of Brucella melitensis and Brucella abortus. The Columns representative of number of the strains, geographical origin, the human and animal sources.

Figure 2

Minimum spanning tree for Brucella isolates using MLVA-16 data.