

DNA polymorphism investigation among local *Brucella* isolates

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Abstract

The present study was undertaken to investigate the deoxyribonucleic acid (DNA) polymorphism finger printing among DNA of local *Brucella* isolates which is a new method in molecular epidemiological studies. It was performed on 125 different human and animal samples obtained from many locations in Baghdad. A total of 11 local *Brucella* isolates were obtained through the study. Its biochemical characters revealed that 8 of them were belong to *Brucella melitensis* and 3 to *Brucella abortus*. All isolates were submitted to genomic organization detection which revealed the presence of two chromosomes, no plasmids were detected. The PFGE analysis after genomic digestion with restriction enzymes to the 11 local *Brucella* isolates and 2 reference strains were done by using two low-cleavage–frequency restriction enzymes (*Not1* and *Xba1*) and one high – cleavage-frequency restriction enzyme which was *Eco R1* followed by pulsed-field gel electrophoresis (PFGE), which revealed as general great similarity in bands patterns (DNA profiles), although the restriction pattern using *Xba1* showed some missing bands in *Brucella abortus* isolates while it is present in *Brucella melitensis* which maybe a marker for species specific detection. Results of *Brucella* genomic digestion with *Not 1* RE showed few and large fragments which were identical from strain to strain, while *Eco R1* showed high number of fragments which were very close to each other and the bands pattern showed great similarity. From this study the following conclusion can be made. The restriction enzymes pattern applied to the whole genome maybe not the perfect way to investigate *Brucella* DNA polymorphism, and we recommend the use of restriction fragments length polymorphism to special genes and not to the whole genome.

Keywords: *Brucella*, DNA profiles, Human, Animals.

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تحري التعدد المظهري لـ DNA عزلات البروسيلة المحلية

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الخلاصة

استهدفت الدراسة الحالية تحري التعدد المظهري (البصم الوراثية) لدنا عزلات البروسيلة المحلية والتي تعد وسيلة جديدة تم اعتمادها في الدراسات الوبائية الجزيئية. تم استخدام 125 عينة من الإنسان ومن مصادر حيوانية مختلفه جمعت من عدة مناطق في بغداد وقد تم الحصول منها على احدى عشر عزلة بروسيلا محليه، التتميط الحيوي لهذه العزلات اظهر ان ثمانية منها كانت من نوع *Brucella melitensis* وثلاثة من نوع *Brucella abortus*. الدنا المنقى من هذه العزلات ومن اثنان مصدرية خضع لفحص طريقة تنظيم الجينوم، بالترحيل باستخدام تقنية Pulsed-Field Gel Electrophoresis (PFGE). ومن ثم تم مقارنته بعد هضمه او تقطيعه باستخدام الأنزيمات القاطعة والتي كانت لأثنان ذوا تكرار واطيء هما (*Not1* و *Xba1*) وواحد ذا تكرار عالي هو *EcoR1*. أظهرت النتائج ان لأحدى عشر عزلة بروسيلا محليه والتي تم الحصول عليها من خلال الدراسة، اثنان من الكروموسومات ولوجود للبلازميدات. اما التحليل باستخدام PFGE وبعد تقطيع الجينوم بالانزيمات القاطعة اظهر بصورة عامة تشابه كبير في نمط توزيع

الباندات (DNA profiles). على الرغم من ان التقطيع باستخدام انزيم *Xba1* قد اظهر وجود بعض الباندات المفقوده في جراثيم البروسيللا المجهضه بينما هي موجوده في جراثيم البروسيللا المالطيه والتي قد تعد علامه لتحديد النوع فيما عدا ذلك كل الحزم متشابهه. اما التقطيع باستخدام انزيم *Not1* فقد نتج عنه عدد قليل من الباندات الكبيره والمتشابهه. بينما اظهر التقطيع باستخدام انزيم *EcoR1* مجموعه كبيره من الباندات المتشابهه والقريبه من بعضها الاخر. نستنتج من هذه النتائج ان طريقة التقطيع بالانزيمات القاطعه والمطبقه على كل الجينوم قد لاتكون هي الطريقه المثلى لدراسة التعدد المظهري لدنا عزلات البروسيللا، ونوصي بطريقه التقطيع والترحيل لجينات خاصه وليس لكل الجينوم.

Introduction

Brucellosis constitutes a major health and economic problem in many parts of the world, including our country (1). It caused by the *Brucellae* which are a family of small, Gram-negative coccobacilli. The disease is zoonosis, transmittable to humans shows a high degree of morbidity. More than 500.000 new cases of human brucellosis are reported each year and according to the World Health Organization (1997), this figure is underestimates magnitude of the problem.

Six species are currently recognized within the genus *Brucella*: *B. melitensis*, *B. abortus*, *B. suis*, *B. neotomae*, *B. ovis* and *B. canis*. This classification is mainly based on differences in pathogenicity and in host preferences (sheep and goats, cattle, swine, desert rat, rams and dogs respectively). Although the six species can be differentiated by conventional phenotypic tests, these species display a high degree of DNA homology in DNA-DNA hybridization assays (>90% identity). Therefore it has been proposed that the *Brucella* genus should comprise only one species i.e. *B. melitensis* and that the other species should be considered as biovars (2,3). However several molecular genotyping methods have been used to show that *Brucella* species display significant DNA polymorphism allowing the species to be differentiated thereby justifying the current species classification. This is also true for the recent marine mammal *Brucella* isolates for which two new species names have been proposed, i.e. *B. pinnipediae* and *B. cetaceae* (4,5).

The presence of two independent chromosomes in *Brucella melitensis* 16 M genome was noticed since 1993. Now it is well known that the genomes of the classical *Brucella* species and their biovars have two chromosomes with the exception of *B. suis* biovar 3 strain 686 which has a single chromosome.

Identifying the organisms that causes infections to their species and biovars is very important to epidemiological studies and helps the epidemiologists to trace- back infections to their sources. This was based only on growth and biochemical characteristics. Improvements in DNA analysis techniques provide other methods.

Analysis by using restriction endonucleases, for studying the relationships of genomic DNA fingerprint

profiles, has been used to differentiate strains of viruses and bacteria and was used in *Brucella* successfully by (6) who found a relationship between DNA fingerprints, species and pathovars. To the best of our knowledge there is no similar study in Iraq and it may be the first in all the endemic Arab area. The aim of this study was to detect the genome organization and the fingerprint profiles of the local *Brucella* isolates.

Materials and methods

Sample collection and its treatments

Blood samples

Fifty humans blood samples were collected from patients with serological (RBPT+ve) and clinical evidence of brucellosis, obtained from different Baghdad regions which were Al-Saheed Al-Sadder Hospital, Baghdad Al-Jadedda. Bab-Al-Muathem and Palestine street privet laboratories, and twenty animal blood samples were collected from animals which shows evidence of brucellosis (with history of abortion) from Al-Fudeliya region in Baghdad. Five ml blood samples volume were withdrawn from each person or animal, injected into prepared sterile trypticase soy broth with 2% sodium citrate and incubated for 48 hours at 37°C, then it was sub cultured on duplicate agar plates and incubated one in air and the other in an atmosphere containing an added 5-10% carbon dioxide.

Aborted fetuses

Five aborted animal fetuses were used, two of them were aborted sheep fetuses taken from a farm in Abo-Gribe, 3 from Al-Fudeliya region, infact that 2 were aborted buffalo fetuses and 1 was an aborted horse fetus. Stomach contents which were withdrawn by a sterile syringe were spread over the surface of an trypticase soy plates with an inoculating loop while tissues from the liver, lung and spleen were rubbed over the surface of the medium, after they were sliced with sterile scissors dipped in ethanol and flamed. The specimens were cultured on duplicate agar plates as described before.

Cheese samples

Fifty local manufactured cheese samples were collected from different markets of Baghdad and treated by take 10 g

of the cheese sample and put it in a sterile bag, then 90 ml of sterile and warmed to (45 °C) 2% sodium citrate solution was added and homogenized in a stomacher for 5 minutes. Milk products such as cheese are generally too heavily contaminated so selective media with mixture of antibiotics and enriched with 5% of fetal calf serum were used. The antibiotics mixture and the serum were added after the media was autoclaved and cooled to nearly 45 °C, then 0.25 ml from the homogenized cheese solution was withdrawn by a sterile syringe and spread over the surface of the trypticase soy agar plates with an inoculating loop. They were cultured on duplicate agar plates and incubated as described before and advised by (7).

Pulsed-Field Gel Electrophoresis of chromosomal and endonuclease restricted *Brucella's* DNA

Pulsed-Field Gel Electrophoresis of chromosomal and endonuclease restricted *Brucella's* DNA with a Contour-Clamped Homogeneous Electric Field (CHEF) for Bacterial Genomic DNA Plug Kit: Provided by BIO-RAD Company-USA, and the Gene Navigator System for electrophoresis provided by Amersham biosciences Company- Sweden.

Preparation of agarose embedded DNA (8)

A pure *Brucella* culture inoculated into a sterile trypticase soy broth and left to grow until it reach the appropriate concentration (5×10^8). Two percent clean cut agarose solution was melted by hot water bath then equilibrated at 50°C during that the bacterial cells was harvested by centrifugation and washed by PBS then the cell's pellet was resuspended by 0.5 ml cell suspension buffer and equilibrated to 50°C. Then the cell suspension was combined with 0.5 ml of the agarose (to reach 1% agarose concentration), then it transferred into cooled plug mold, the agarose was allowed to solidified by placing the mold at 4°C for 10 minutes. The solidified agarose plugs were pushed into microcentrifuge tubes containing 0.5 ml lysozyme buffer then 20 µl of lysozyme stock was added then incubated at 37°C for 2 hours. The lysozyme solution removed and the plugs were rinsed with sterile water before 0.5 ml of proteinase K reaction buffer was added followed by 20 µl of proteinase K stock. Then it incubated overnight at 50°C without agitation. The plugs were washed four times one hour each with 1X wash buffer [except the third wash which was with 1 mM PMSF (Phenylmethane sulfonyl fluoride) solution] at room temperature with gentle agitation. The plugs were stored at 4°C until required for enzyme digestion.

Restriction enzyme digestion of plugs (9)

A small portion (nearly 1/3) of the plug was sliced off then placed in a sterile 1.5 ml microcentrifuge tube and washed once for 1 hour in 350 µl of 0.1 X wash buffer, decanted then another sufficient amount of fresh 0.1 X

wash buffer was added to cover the plug. The wash buffer was aspirated off and 350 µl of the appropriate 1 X restriction enzyme buffer was added for about 1 hour with gentle agitation at room temperature. The buffer then aspirated off and the restriction enzyme reaction mixture was added as follows:

- 1- *Not* I Restriction enzyme reaction mixture: [Sterile deionized water (82 µl), RE. 10X Buffer (10 µl), Acetylated BSA (1µl of 10Mg/ µl), Restriction Enzyme (7 µl of 10U/ µl)].
- 2- *Xba* I Restriction enzyme reaction mixture: [Sterile deionized water (84µl), RE. 10X Buffer (10 µl), Acetylated BSA (1µl of 10µg/ µl), Restriction Enzyme (5µl of 12U/µl)].
- 3- *Eco* R1: [Sterile deionized water (82µl), RE. 10X Buffer (10µl), Restriction Enzyme (8µl of 10U/µl), the Acetylated BSA provided with the buffer].

After four hours digestion, the buffer was removed and incubated in 350 µl of 1 X wash buffer for approximately 30 minutes with gentle agitation.

PFGE of the DNA plugs

Briefly the thermostatic circulator was prepared and fixed on 14°C before it was started to cool the circulating buffer for nearly at least one hour before the inserting of the gel support tray with the plug DNA samples slices with it. Then the GN controller was programmed with the run parameters as follows:

- 1- In case of *Not* I restricted DNA plugs: Two phases were used. The first phase run parameters was an 5 sec pulse time for 22 hours at 100 mA and 200 Volt. The second phase was in 10 sec pulse time for 22 hours at 100 mA and 200 Volt.
- 2- *Xba* I: One phase of 50 sec pulse time for 22 hours at 100 mA and 200 Volt.
- 3- *Eco* R1: One phase of 10 sec pulse time for 4 hours at 100 mA and 200 Volt.
- 4- The chromosome DNA was run two times first at 120 sec /20 hours/80 mA /120 Volt second at 10 sec/44 hours/100mA /200 Volt.

Then the gel was removed and stained with 250 µl of the stock ethidium bromide stain solution (1 mg ETBr/ml water). Staining takes up to 30 minutes, destained and visualized on a UV light box.

Results

The results of *Brucella* isolation

Eleven *Brucella* isolates were obtained from different sources as shown in table (1), *Brucella* culture recognized first on the basis of colonial morphology (which was round translucent pale honey color) and slide agglutination test, subsequent examination by routine bacteriological methods was necessary to identify the genus *Brucella* before they

submitted for *Brucella* typing tests, any isolate that was differ in even one test was eliminated from further consideration as member of the genus *Brucella*. So the obtained isolates were Gram-negative, coccobacilli, arranged singly, in pairs, short chain and with small groups, negative for haemolysis on blood agar, and it does not grow nor lactose- fermenting on MacConkey agar, negative for nitrate reduction and indol production, while they were positive for oxidase and catalase tests. The biotyping characteristics of the isolates which were obtained from human blood and aborted sheep fetuses showed that they were negative for H₂S production, positive for urease, and grow well without CO₂ requirement. On the basis of the biochemical results, these isolates were identified as *Brucella melitensis*. While the isolated which were obtained from the aborted buffalo fetus and cheese were positive for H₂S production, positive for urease, and require CO₂ to grow, so on the basis of the biochemical results, these isolates were identified as *Brucella abortus*.

Table 1: Local *Brucella* isolates, its species and source.

<i>Brucella</i> species	Source	The Number
<i>Brucella melitensis</i>	Human	6
<i>Brucella melitensis</i>	Sheep	2
<i>Brucella abortus</i>	Buffalo	1
<i>Brucella abortus</i>	Cheese	2

Genomic fingerprinting of *Brucella* local strains isolated from human and animal sources identified by PFGE

The isolates were submitted to genome organization detection which as expected was found that it consist of two chromosomes, but that only when it was electrophoresis it by PFGE for 44 hours with two phases (5 sec. pulse time phase for first 22 hours and 10 sec. pulse time phase for the second 22 hour), as shown in figure (1).

Investigation the polymorphism among the strains of the local *Brucella* DNA was done by analyzing the PFGE fingerprinting profiles after genome digestion by restriction endonucleases (RE). The choice of the RE was one of the important factors effecting our results. We tried *Eco* RI, one of the high-cleavage-frequency restriction enzymes, which recognize 6 bp sites (5'...G'AATTC...3') and two low-cleavage-frequency RE. *Not* I which recognize 8 bp site (5'...GC'GGCCGC...3') and *Xba* I which recognize 6 bp (5'...T'CTAGA...3') site but yet consider low-cleavage-frequency RE because its frequency in *Brucella* genome is low. So we consider it one of the low-cleavage-frequency RE. in concern with *Brucella*.

Results of genomic digestion with *Eco* RI

The results revealed high number of fragments which were very close to each other and the bands pattern as general showed great similarity.

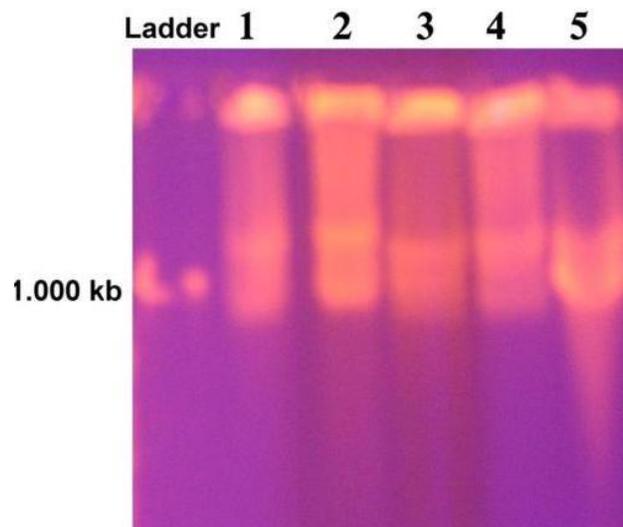


Fig. 1: PFGE profiles of intact genomic local *Brucella* DNA, revealed the separation of its two chromosomes. Gel was run at 200 volt, 100 mA for 44 hours (5. sec. pulse time for 22 hours and 10 sec. pulse time for the second 22 hours). (1, 2, 3, 4, 5) are randomly selected *Brucella*'s DNA.

Results of genomic digestion with *Xba* I

It showed some missing bands in the *Brucella abortus* strains (A5, 38C and 39C) which extracted from aborted buffalo fetus and cheese, while it present in all *Brucella melitensis* strains extracted from human and aborted sheep fetuses which may be a marker for species specific detection.

Results of genomic digestion with *Not* I

The results of *Brucella* genomic digestion with *Not* I RE are shown in figure (2). *Not* I RE is an 8-base-pair recognition site, so it was expected to have few and large fragments, so as expected the results showed nearly seventeen bands on the restriction pattern with great similarity.

Discussion

As PFGE has been considered one of the most useful developments in molecular epidemiology for the past few decades and is now regarded as the gold standard for molecular typing of microorganisms (10,11) and because there no any other previous study that demonstrate *Brucella* local strains fingerprints. This technique was used in the present study for DNA analysis of 11 local *Brucella* strain and 2 reference vaccine strains.

During the course of the study, 11 *Brucella* isolates were obtained, six of them, from patients blood, all were

belong to *B. melitensis*. Which is the most virulent so causes the most clinically apparent disease (12) that forced the patients to attained the clinics or hospitals and not because *B. melitensis* is the most widespread. Brucellosis caused by *B. abortus* is the most widespread in the world (12) and in Iraq (13,14). However (15) also found that *B. melitensis* more prevalent among human brucellosis patients. The other 5 isolates were from animal sources two of them were *B. melitensis* from aborted sheep fetuses and the other three were *B. abortus*, one of them form aborted buffalo fetus and the other two were from cheese samples.

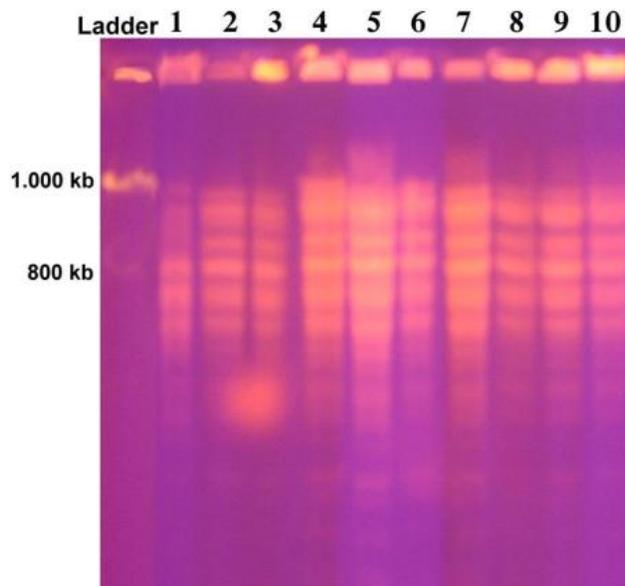


Fig. 2: PFGE profiles of *Not* I –digested local genomic *Brucella* DNA from different sources. Gel was run at 200 volt, 100 mA, 5 sec. pulse time for 22 hours and 10 sec. pulse time for another 22 hours.

These isolates were submitted to genome organization detection which as expected, two chromosomes have been found, but only when we electrophoresed the total intact DNA by PFGE for 44 hours and for two pulse time phases. No plasmid was detected in all our isolates. All biovars of *B. melitensis* and *B. abortus* known to have two chromosomes also *B. suis* biovars 1, 2 and 4 with the exception of biovar 3 which contains only one chromosome (16). When we first electrophoresed the genome for 20 hours and one pulse-time phase the genome did not enter the gel. This may be the reason why (17) said that circular molecules could not enter the gel. In any event the local *Brucella* chromosomes did not move much, we can see them trapped near the wells although they left for 44 hours (figure 1). We compared local *Brucella* chromosomes with the high-molecular –weight lambda marker but we can not depend on it much because as (18) said, in the very large

fragments the degree of accuracy is rather low. This is may be the reason why we can see them very close to each other in spite of the difference in size in nearly 1 Mbp (figure 1).

Restriction endonuclease analysis of the *Brucella* local strains DNA with the high-cleavage-frequency *Eco* R1 enzyme, has not allowed a clear distinction among *Brucella* local strains. There were some high-molecular- weight bands missing and there were some differences in the thickness and intensity of some bands which may be due to differences in DNA concentration in the samples. As general, no important genetic differences were detected. This was observed previously by others (6) number of fragments obtained by *Eco* R1 digestion or of the strong homogeneity of the genus (3) and this is why (19) concluded that the commonly used restriction endonucleases such as *Eco* R1 or *Hind* III demonstrate only minor differences between *Brucella* strains, so it was unsuccessful for typing when applied to the whole genome.

The PFGE following digestion of *Brucella* genomic DNA with *Xba*I in one phase pulse-time and 22 hours appear to be not sufficient to reveal a full separation of genomic DNA fragments, although (20) used the same run parameters, but for *V. cholerae* and *Salmonella enterica* respectively and they had a full separation.

Actually *Brucella* spp. characterized by high G+C content over 57% (21) and this was found to have a negative effect on the migration across the gel (22) and according to (18) the migration distance in the gel depends on not only the pulse time but also the G+C content of the molecule. Nevertheless we had enough differences in the electrophoretic gel pattern that allowed us to discriminate between *B. melitensis* and *B. abortus*. These results agree with the results of (6) which revealed a relationship between DNA fingerprints and species. However we can see a great similarity within and between species. This is may be the consequence of the high homogeneity with all members showing > 95% homology in DNA-DNA pairing studies. Thus classifying *Brucella* as a monospecific genus (2). For this reason, we tried to digest DNA with another low-cleavage-frequency endonuclease which was *Not* I.

This time two phases pulse-time was used, and for 22 hours each, (5 sec. first 22 hours and 10 sec. pulse time for the other 22 hours), thus full separation of DNA fragments was obtained. But unexpectedly the DNA fingerprints pattern in all local *Brucella* DNA were identical. Our results in contrast with Allardet (6) showed less DNA fragments (nearly 17) and the DNA fingerprints patterns were identical. In our study we analyzed the DNA of the local *B. melitensis* and *B. abortus* isolates only, while the above mentioned study used all species of the *Brucella* reference strains, so they expected to have some heterogeneity in DNA patterns. Beside it was observed by other researchers that *B. melitensis* and *B. abortus* DNA signatures were similar (23) and according to (21) *B.*

abortus shared more fragments and had fewer nucleotide polymorphisms with *B. melitensis* than with other *Brucella* spp.

Genetic relatedness within *Brucella* has been studied by Benjamin (3) using multilocus enzyme electrophoresis (MLEE) of 99 *Brucella* isolates, including the type strains from all recognized species. Their results revealed very limited genetic diversity, thus they support the proposal of Verger *et al.* (2) of a monospecific genus. However some researchers tried to use other molecular genotyping methods like polymerase chain amplification of selected sequences followed by restriction analysis which has provided an evidence of polymorphism in a number of genes including *omp2*, *dnak*, *htr*, and *ery* (24).

From the above study we can conclude that all the *Brucella* local strains obtained from different sources (human, animals and cheese) have two chromosomes, which show very similar band patterns (fingerprints) after digestion with the restriction enzymes (*EcoRI*, *XbaI* and *NotI*) which applied to the whole genome.

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