

Different Dna Extraction Techniques from *Brucella melitensis* 16M

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Abstract: Detection of *Brucella* species using nucleic acid techniques requires efficient unbiased DNA extraction procedures. In this study, we compared six protocols for DNA isolation from pure culture of *Brucella melitensis* 16M and the efficiency of different extraction processes were evaluated using Polymerase Chain Reaction (PCR). These protocols included boiling, the use of CTAB phenol chloroform extraction, TritonX-100, Chelex 100 with or without proteinaseK and commercial kit. There were some differences between the various DNA extraction methods. The CTAB method seems to have the highest genomic DNA yield with the highest quality for DNA techniques compared to other protocols.

Key words: Brucella • DNA extraction • DNA purification • PCR

INTRODUCTION

Brucellosis is one of the most important widespread zoonosis which is still responsible for economic losses of livestock worldwide [1, 2]. In cattle, the disease is belonging to reproductive problems causes significant economic loss by causing a decrease in calving through abortion and reduction in meat and milk production [3, 4]. The causative organisms of brucellosis are Gram-negative facultative intracellular pathogens that may affect a range of different mammals including man, cattle, sheep, goats, swine, rodents and marine mammals and in most host species. To date, many research works have been done to reduce the widespread of brucellosis through standardization and implementation of diagnostic methods [5, 6].

Currently, bacterial cultivation is the “gold standard” of laboratory diagnosis, this requires prolonged incubation, blind subcultures and special growth media due to their comparatively long doubling time. *Brucella* species grow slowly on primary cultures and subcultures, while their inert biochemical profiles hamper fast identification of isolates; however this technique has low sensitivity, ranging from 15 to 70%. Consequently, detection and identification of *Brucella* species in clinical specimens by cultures may still be a

difficult task with significant delays and hazards to lab personnel [7] as *Brucella* species are class III pathogens [8].

The disease constitutes a serious infection in both human and animal necessitating treatment with a prolonged course of antibiotics; moreover, accuracy and short turnaround time are required for the diagnostic tests [9].

Thus, diagnosis is usually based on indirect serological tests, including several agglutination tests. Broad range of test sensitivity, low specificity in endemic areas, lack of useful techniques to diagnose chronic disease and relapse, presence of cross-reacting antibodies, inability to distinguish between active and inactive infection and lack of timeliness constitute problems associated with brucellosis serology [10-12]. To overcome some of these problems, at least two serological tests have to be combined to avoid false negative results.

Due to the tremendous progress in molecular pathology during the last decade, molecular techniques are moving rapidly from research to be routinely used in pathological diagnostics. As for other fastidious pathogens, molecular methodology offers an alternative way of diagnosing brucellosis. Nucleic acid amplification techniques, like PCR which is highly useful for bacterial

detection and identification besides being characterized by short turnaround time can overcome the limitations of conventional methodology. DNA extraction is the first step and is important because DNA concentrations and purity can differ according to the extraction method. Thus, the final diagnostic efficiency is influenced by it.

The present study addresses the issue of comparing six reported extraction techniques for DNA isolation and purification from *Brucella* cells and select the most appropriate DNA extraction method yielding the highest quality and quantity of isolated DNA, robustness of results as well as the ease of implementation.

MATERIALS AND METHODS

Bacteria: The reference strain used in this study was *Brucella melitensis* 16M, which was kindly supplied by the Department of Production of Antigens and Sera (Veterinary Serum and Vaccine Research Institute). It was identified by biochemical tests such as positive oxidase, urease test, negative hydrogen disulphide production, no requirements for carbon dioxide and growth in the presence of basic thionin and fuchsin (20µg/ml) as well as agglutination by monospecific antisera.

Preparation of Bacterial Cell Suspensions: Freshly cultured *Brucella melitensis* 16M, was prepared as follows: bacterial concentration was adjusted to an optical density (OD) of (1.0) at 540 nm, killed by the addition of 70% methanol in sterile saline (0.9%NaCl) and recovered by centrifugation at 5000 rpm for 5 min. Triplicate samples were washed twice with 5 ml of sterile phosphate buffer saline, then recovered by centrifugation at 6000xg for 5 min. Each pellet was adjusted to contain about 10⁹ CPU and then re-suspended in different solutions according to the method used.

DNA Extraction: DNA was extracted from bacterial cells by the following extraction methods.

Method 1: Extraction by Boiling: According to Soumet *et al.*, 1994, the washed adjusted pellet was re-suspended in 100µl of sterile double distilled water and placed in a boiling water bath for 15 min. Upon cooling to room temperature, tubes were centrifuged at 4000 rpm for 5 min.; 1µl of 10mg/ml RNase was added.

Method 2: CTAB Phenol Chloroform Extraction: According to method modified from Ausubel *et al.*, the washed adjusted pellet was re-suspended in 567µl of Tris-EDTA (TE) buffer (50 mMTris-Cl, 125 mM EDTA [pH 8]).

Then, 30µl of 24% sodium dodecyl sulphate (SDS) and 3µl of proteinase K (20mg/ml) were added. The re-suspended cells were incubated for one hour at 37°C; 100µl of 5M sodium chloride was added and mixed thoroughly. Then 80 µl CTAB/NaCl (4.1 g NaCl, 10 g CTAB in 100 ml H₂O) solutions were added, mixed thoroughly and incubated for 10 min. at 65°C. One volume (0.7 to 0.8 ml) of 24:1 chloroform/isoamyl alcohol was added, mixed thoroughly and centrifuged for 4 to 5 min. The supernatant was transferred to a fresh tube and 1 volume of 25:24:1 phenol/chloroform/isoamyl alcohol was added with thorough extraction and centrifuged for 5 min. The supernatant was transferred to a fresh tube and 0.6 volume of isopropanol was added and mixed gently. After brief centrifugation, supernatant was discarded and 70% ethanol was added to the pellet. After further centrifugation, pellet was dried and re-suspended in 100µl TE buffer.

Method 3: Triton X-100

According to Wang *et al.* [15], the washed adjusted pellet was re-suspended in 1.5ml of sterile phosphate buffered saline (PBS, 0.05mol l⁻¹, pH 7.4) and centrifuged at 9000g for 3 min. The pellet was washed three times with PBS, once with water and then re-suspended in 50µl water. The suspension was diluted 1:10 with 1% Triton X-100, incubated in a boiling water bath for 5 min., then immediately cooled in ice water.

Method 4: Chelex-100 with Proteinase K: DNA was extracted as previously described by Walsh *et al.* [16] and modified by [17, 18]. Only 500 µl 10% Chelex-100 slurry were prepared. The pellet of 200µl *Brucella* culture was mixed with the prepared 500 µl of 10% Chelex-100 resin and 10 µl of 20 mg/ml proteinase K were added and incubated at 56°C for 60 min. and the mixture was boiled for 15 min. only 2.5volumes of ice cold 100% ethanol were added and centrifuged at 13,000 g for 10 min. at 4°C and the supernatant was discarded. The pellet was washed with ice-cold then with 70% (vol/vol) ethanol and centrifuged again at 13,000g for 10 min. at 4°C. The supernatant was removed and the DNA pellet was dried and re-suspended in 100 µl DDW.

Method 5: Chelex-100 Without Proteinase K: The procedure was performed as previous method (No.4) without adding proteinase K.

Method 6: QIAamp DNA-blood-mini-kit Extraction (Commercial Kit with Catalogue Number, 51104): This method was performed following the Qiagen protocol

provided by the manufacturer. The washed adjusted pellet was mixed with 200 μ l AL buffer, homogenized and incubated for 10 min. at room temperature. Only 200 μ l of 96% ethanol (Merck, Germany) were added. The mixture was transferred to a QIAamp column and centrifuged for 1 min. at 8,000 x g. The column was put in a new collection tube, only 500 μ l AW1 buffer were added and centrifuged for 1 min. at 8,000 rpm. This procedure was repeated with 500 μ l AW2 buffer and the column was centrifuged for 1 min. at 14,000 xg. To remove all ethanol from the column it was put in a new collection tube and then subjected to a dry spin for 1 min. at 14,000 rpm. Elution was performed by adding 200 μ l EL buffer, incubated for 5 min. at room temperature followed by centrifugation for 1 min. at 8,000 xg.

DNA Concentration and Purity Determination: The DNA concentration of the extracted DNA was determined using Nano Drop® Spectrophotometer; all assays were performed according to the manufacture instructions using 1 μ l of extracted DNA for each reaction. The concentration of DNA was calculated based on the approximation that an absorbance reading of 1 μ l of the purified DNA at 260nm was taken to correspond to 50ng/ μ l. DNA purity was estimated by determination of the A260/A280 ratio and reference value for purity was found to be 1.8.

Testing DNA Quality Using PCR Technique: PCR was performed in a 50 μ l volume reaction mixture containing 10 mM Tris-HCl (pH 8.4), 1 mM MgCl₂, 50 mM KCl, 200 μ M each deoxyribonucleotide triphosphate (dATP, dGTP, dTTP and dCTP; Promega, Madison, WI, USA), 0.5 U of Taq polymerase (Promega, Madison, WI, USA), 10 nM of each primer (BioTeZ Berlin-Buch GmbH, Germany) and 5 μ l extracted DNA. The primers (forward primer, 5' AAATCGCGTCCTTGCTGGTCTGA; reverse primer, 5' GACGATAGCGTTTCAACTTG) described by Bricker and Halling [19], were used to amplify a target sequence of 731-bp specific to IS711 gene of *B. melitensis*. PCR was done with a thermocycler (Primus 25 advanced®, PEQLAB Biotechnologie GmbH, Germany) as the following procedure: 30 cycles of PCR, with one cycle consisting of 120 seconds at 95 °C for DNA denaturation, 45 seconds at 45 °C for DNA annealing and 30 seconds at 72 °C for extension. The last cycle included final extension of the sample at 72°C for 6 min. The PCR products were determined by electrophoresis on 2% agarose gel with ethidium bromide (0.5 mg/ml).

Statistical Analysis: The data were analyzed by the least squares technique using General Linear Models (GLM) procedure of SAS software version (8) (SAS 2004) [20] to detect differences between various methods of extraction used [21].

RESULTS AND DISCUSSION

In our present study, we estimated many diverse methods of extracting *Brucella* DNA in order to improve its quality and quantity. To achieve this task, several different DNA extraction methods were compared for whole DNA extraction and purification from *Brucella melitensis* 16M. Extraction methods were chosen based on their ability to produce DNA extracts with a range of quantity and quality that represented general classes of lysis and purification. Ongoing methods for isolation and purification of DNA from bacteria broadly include lysing the cells and inactivating DNase using special salts and non-ionic surfactants. The liberated DNA is then precipitated from the solution. So, the present work has evaluated six different protocols for extraction of DNA to know which method is estimated to produce high yields of DNA with minimal contaminants from *Brucella*.

Qualitative and quantitative analysis of all the three replicates of DNA samples extracted from *Brucella melitensis* 16M by various extraction protocols by using NanoDrop® Spectrophotometer, are shown in the Figures 1-6 and Table 1.

The ratio of absorptions at 260nm vs 280nm is commonly used to assess DNA contamination with protein solutions, since proteins (in particular, the aromatic amino acids) absorb light at 280nm. Relative qualitative analysis of different DNA extraction methods applied to all the three replicates was calculated automatically but based on subtracting the Abs_{260/280} of DNA samples from the value of 1.8, which was the purity value of DNA and taken as the reference of purity. So, values which are more or less than 1.8 indicate impurities in DNA, in other words the samples could be contaminated mainly with protein [22]. As the ratio of absorptions at 260nm vs 280nm indicates the DNA contamination with protein, the ratio of absorptions at 260nm vs 230nm is used to assess carbohydrates contamination of DNA which should be more than 2.0 [23]. Culture extracted by CTAB phenol chloroform extraction method yielded mean values higher than 1.8 (1.927) indicating trace contamination with protein, so the DNA obtained by this method is considered pure enough.

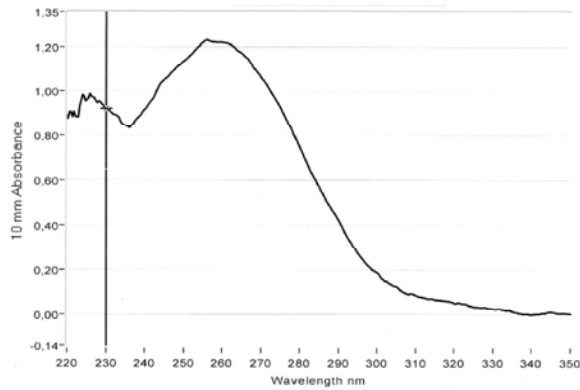


Fig. 1: Extraction of DNA by using boiling

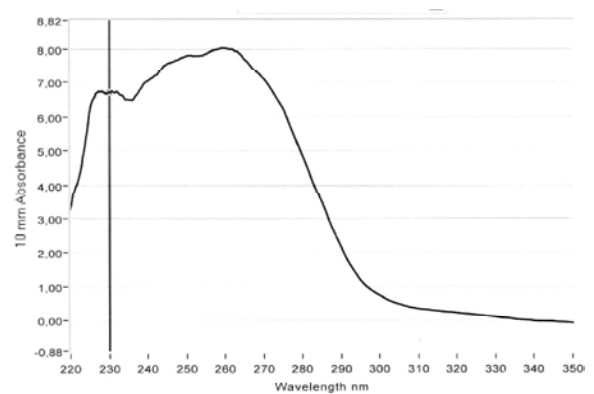


Fig. 4: Extraction of DNA by using Chelex-100 with proteinase K

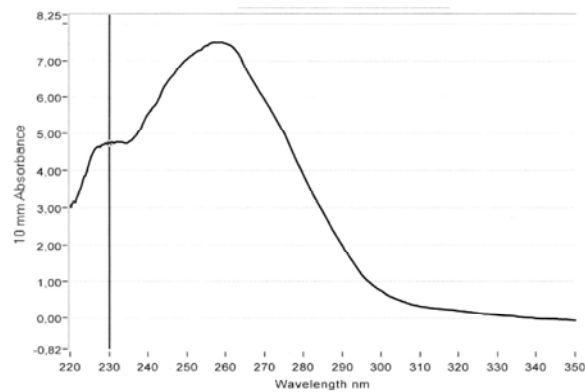


Fig. 2: Extraction of DNA by using CTAB

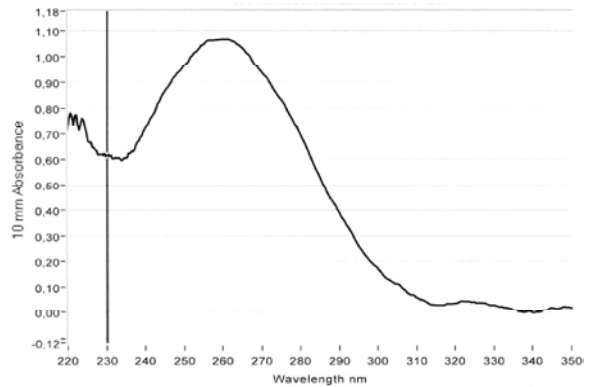


Fig. 5: Extraction of DNA by using Chelex-100 without proteinase K

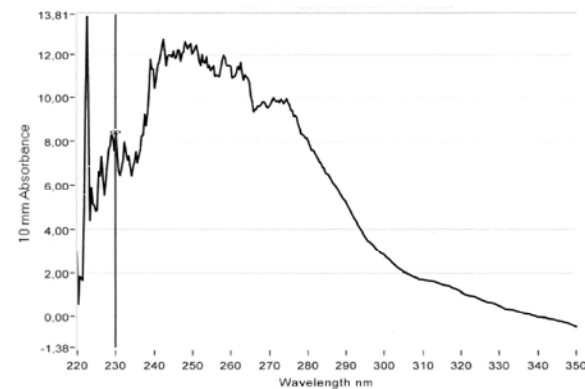


Fig. 3: Extraction of DNA by using TritonX-100

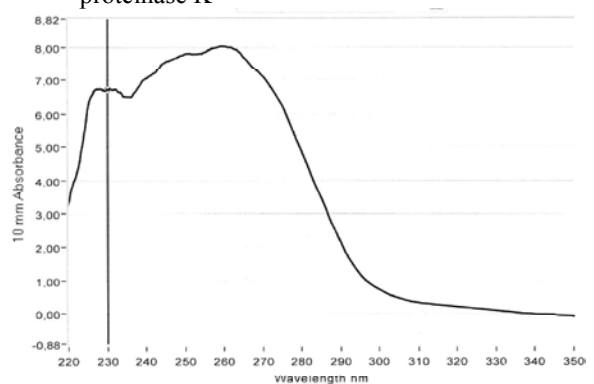


Fig. 6: Extraction of DNA by using QIAamp DNA-extraction kit

The results obtained by this method were followed by chelex 100 with proteinase K, QIAamp DNA extraction kit, Chelex 100 without proteinase K and tritonX-100 methods where the ratio of 260/280 was 1.657, 1.627, 1.551 and 1.429, respectively. Notable, QIAamp DNA extraction method may be was misjudged in sensitivity due to the elution was done in 200 μ l instead of 100 μ l as was done in CTAB phenol chloroform method. Although boiling yielded mean values of 1.630, this

method cannot be regarded reliable for extracting complete genomic DNA for the purpose of being used in biochemical tests.

From these result, all methods except CTAB phenol chloroform extraction method, have ratio less than 1.9 which could indicate protein contamination due to decreasing protease activity.

Table 1: Parameters used to evaluate the extracted DNA yields and quality of *Brucella melitensis* 16M.

Purity of DNA at Abs 260/280		Concentration of DNA, (ng/μl)		Absorbance		Method
±SE	Mean	±SE	Mean	±SE	Mean	
0.001 a	1.927	5.745 a	572.133	0.003 a	8.436	CTAB
0.0009 b	1.657	1.559 b	400.900	0.004 b	6.701	Chelex with proteinase K
0.001 d	1.551	2.021 d	53.500	0.002 e	0.615	Chelex without proteinase K
0.0006 e	1.429	7.507 c	373.167	0.005 c	4.743	TritonX-100
0.004 c	1.63	3.464 d	61.000	0.004 d	0.924	Boiling
0.0007 b	1.627	1.339 b	400.300	0.003f	5.944	QIAamp DNA- extraction kit
**	**	**	S.O.V.			

P<0.01 Highly significant (**)

Means with the same letter in the same column are not significantly different.

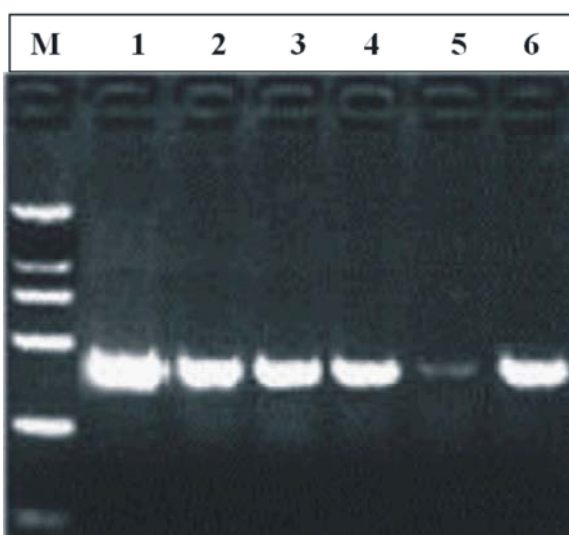


Fig. 7: Agarose gel electrophoresis showing the PCR product of IS711 gene from DNA extracted by CTAB (lane 1), Chelex with proteinase K (lane 2), boiling (lane 3), Chelex without proteinase K (lane 4), Triton X (lane 5) and QIAamp (lane 6) while (M) is the marker lane.

Table 1, shows the yield of *Brucella* DNA extracted using different protocols. Results showed that the CTAB method produced the highest yield of DNA, producing an average of 572 μg DNA followed by Chelex with proteinase K method which produced 400 μg DNA/g

Typical PCR amplifications were found to produce a DNA band of the expected size 731 bp by the six template preparations of the same concentration as shown in Fig 7. Although all methods showed amplification, some differences in the intensity of the specific band (Fig 7, lanes 1-6) and background pattern were observed. In the extraction method 2, using Cetyltrimethyl ammonium bromide (CTAB), a cationic detergent, has been proved as good denaturant in the preparation of nucleic acids and

has the ability to precipitate polysaccharide materials and proteins from the cell wall of bacteria as *Brucella* in the presence of sodium. Hence, remove most PCR inhibitors therefore a significant and specific PCR product was detected rather than gram negative bacterial cell membranes are very sensitive to Tris and EDTA solutions but in case of *Brucella*, it is resistant to non ionic detergent as EDTA but is more sensitive to the action of ionic detergent as SDS. So, we used high concentration of EDTA. The CTAB phenol-chloroform step at the end of the extraction of this protocol improves the protein purity which agrees with some previous studies [24, 25].

In contrast, method 3, was not reliable enough and a weak band was noted. Triton X-100 is non-polar detergent is milder solubilizing agent, it seems to have much more limited ability to initiate the disruption of the bacterial cells.

Methods 1, 4, 5 and 6 showed nearly the same amplified PCR product. Boiling may destroy some PCR inhibiting factor that is not removed in other extraction procedures or the addition of Chelex-100 who acts as a chelating resin for metallic polyvalent ions in addition to its other roles in DNA purification [26] and with or without proteinase K, a serine protease produced by the fungus *Tritirachium album*, which cleaves adjacent to the carboxyl groups of aliphatic and aromatic amino acids involved in peptide bonding, including those comprising the peptide cross links inter-bridges of the peptidoglycan layers of the cell walls of bacteria [27]. Likewise, QIAamp DNA kit was effective as other extraction methods.

There were differences in the time used for each DNA extraction method. Boiling was the most rapid extraction method taking about 20 min, TritonX-100 (35 min., Chelex -100 without proteinase K (55 min.), Chelex-100 with proteinase K was the same as QIAamp DNA kit (120 min.). Using CTAB phenol chloroform extraction method took the longest time (200 min.).

DNA extraction is a multi-step process; high sensitivity detection assays require DNA extraction methods with high efficiency. DNA extracts must meet application requirements and DNA quantity is an indicator of extraction efficiency and quality parameters

It is concluded that among the six analyzed DNA extraction methods, CTAB phenol chloroform extraction method meet our aim of the present study providing DNA sufficient for any molecular biology application where it is practical, cost effective, with short turnaround time and limited hazards to lab personnel as well as being simple enough to be routinely done. The extract methods presented here can serve as a starting point for the development of a standard procedure for evaluating DNA extract quality.

ACKNOWLEDGEMENT

The present study was achieved in the frame of PROCAMED project, supported by the European Union (Trans frontier cooperation in the frame of European Neighbourhood and Partnership Instrument-Joint Operational Programme of the Mediterranean Basin (IEVP-CT), managed by the Joint Managing Authority-Autonomous Region of Sardinia(Italy) and coordinated by CIRAD-France. Many thanks for the help of Prof. Dr. SayedAbd El-Baki.

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