Evaluating the bacterial culture technique by Polymerase chain reaction for the diagnosis of *Brucella abortus* in milk of cows suspected with brucellosis.

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Summary

The aim of this study is to determine the sensitivity of bacterial culture technique in the detection of *Brucella abortus* in milk samples of aborted cows. Sixty samples of milk were collected from aborted cows during a period which did not exceed two months after the abortion. All of them were positive for rose bengal test. Results showed that *Brucella abortus* was isolated from 7 out of 60 (11.6%) from the milk of aborted cows, while PCR test showed that 32 out of 60 (53.3%) milk sample contained *Brucella abortus*. The specificity of culture techniques was 10%, but its sensitivity was only 21.8%. Beside the cautions in dealing with live *Brucella abortus* (as culture), it is also less sensitive than PCR, though it is better to use PCR technique in the diagnosis of brucellosis in aborted cows milk.

Keywords: *Brucella abotrus*, Milk, PCR, Cow, Brucellosis.

Introduction

Brucellosis is caused by *Brucella* spp. which is composed of many species which Include: *B. abortus, B. melitensis, B. suis. B. ovis, B. canis, B. neotomae, B. microti, B. inopinata, B. ceti* and *B. pinnipedialis* (1). Brucellosis is still one of the most common bacterial zoonosis in the Mediterranean region (2). Most of the countries that are faced with the economic losses and public health issues caused by animal brucellosis have governmental programs for the eradication or control of the disease. Accurate diagnostic procedures are critical for the success of these programs (3). The gold standard for the diagnosis of brucellosis is isolated and identification of the Brucella species, requires high security laboratory facilities and highly skilled technical personal to handle (4). Because of their potential to detect very small numbers of Brucella, PCR –based assays have been applied recently to diagnose many infectious diseases. PCR assay has been shown to be a valuable method to detect DNA from different microorganisms. Although there are several studies of Brucella DNA detection by PCR with pure cultures (5-8), few studies have been performed with clinical or field samples and little comparisons with bacteriological have been made (9). The aim of this study was to determine the sensitivity of bacterial culture technique in the detection of *Brucella abortus* in milk samples of aborted cows.

Materials and Methods

Sixty milk samples were collected from cows (in period test January to May in 2014) suffering from abortion and were positive for Rose Bengal during the period not exceeding two months after abortion. The udder was washed and the teats were disinfected and dried using alcohol (10), then the first drops of milk were ruled out and 10 ml of milk samples collected directly into sterile plastic tubes. The samples transported as soon as possible to the laboratory. Samples were centrifuged at 1000r.pm (10 minutes) at 4° C and the fatty material was separated from the rest of the components of milk. Brucella was detected according to the method mentioned by (10) Brucella Basel agar, (Biolive_Italy) was used and 5 % of sterile horse blood was added to the agar and one ampule of (Brucella selective supplement, HIMEDIA-INDIA) was added for each 500 ml of media. Aloop full from the fatty layer and another from the deposit were used to inoculate Brucella basel agar and incubated for 5 days at 37 °C. The plates examined in order to detect colonies suspected of Brucella (with a soft appearance pearly white) and was purified by taking single colony and inoculated again in the same circumstances of initial culture. Gram stain and modified Ziehl-Neelson stain of the suspected colony was done. The Brucella bacterium diagnosed according to colonial characteristics and bacterial morphology of

stained smears and biochemical test (11). Then the isolated bacteria diagnosed using Polymerase chain reaction (PCR) techniques. PCR test; was applied by two methods the first one from cultured bacteria; boiling method followed to extract the DNA template. One colony of isolated bacteria dissolved in 200 micro litter distilled water in epindurf tube (capacity 1.5 ml). The tubes put in water bath at 100°C for 10 minutes, then put directly in ice. The tubes centrifuged at 12000 rpm for 20 second at 4°C. The supernatant (which contain the DNA) put in epindurf tubes and stored at -20 till used in preparation of reaction mixture (1).

The second method is by direct separation of DNA from milk samples: The DNA separated according to the method described by (12), by mixing 500 micro litter of milk sample with 100 micro litter of NET buffer (which prepared by mixing 50 mM NaCl- 125 mM EDTA- 50 mM Tris-HCL) and 85 micro litter of 24% SDS solution, and the mixture incubated at 80 °C for 10 minutes. The mixture left to cool on ice for 10 minutes, then 20 micro litter of Proteinase K enzyme was added to the mixture and incubated at 56 °C for 12 Hours. DNA templates isolated using standard protocol of Phenol-Chloroform-Isoamyl alcohol, PCI. All components used in preparation of reaction mixture put in ice and the mixture prepared as in (Table, 1).

Table, 1: Compounds used in preparation of Reaction Mixture.

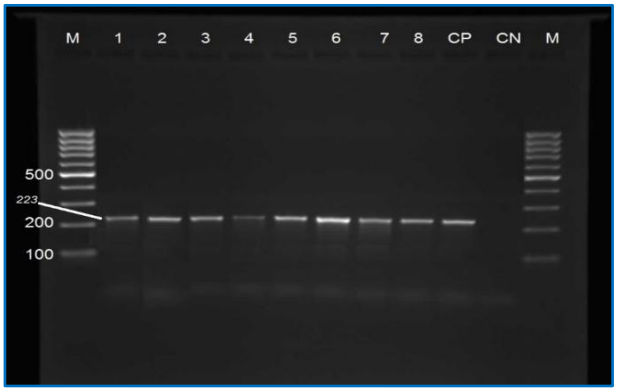
Compounds used in preparation of Reaction Mixture	Amount
Taq PCR Master Mix KIT (Qiagen, Germany) Which contain Taq DNA Polymerase (2.5 Unit), PCR Buffer with 3mM MgCL2, 200µMdNTP (Qiagen, Germany).	25
Primer A (B. A. Forword) 5/ ACG, CAG, TCA, GAC, GTT, GCC, TAT,3/ (Funakoski, Japan)	0.3 from 100pM Solution
Primer B (B.A. Reverse) (BCSP31) 5/ TCC, AGC, GCA, CCA, TCT, TTC, AGC, CTC, 3/ (Funakoski, Japan)	0.3 from 100pM Solution
DNA Template	3
DNA free water (Qiagen, Germany)	21.4
Total	50

Results and Discussion

The results of bacterial culture revealed that 7 out of 60 (11.6 %) milk samples collected from aborted cows contained Brucella which were diagnosed according to morphology, cultural characteristics, and biochemical tests. Brucella isolates appeared as gram-negative bacteria, and staining by modified Ziehl-Neelson stain, the colony grow on Brucella Basel agar after 5-7 days as smooth convex colony, pale yellowish in color (droplet honey). Brucella isolates gave positive results in catalase test, oxidase test, H_2S production test and urease test whereas it gave a negative results in Methyl red test, Voges-Proskauer test and indol test. There is no growth on MacConky Agar but it growth in blood agar without any type of hemolysis. This characteristics was matching with Brucella characteristics that recorded by (1, 10 and 11) (Fig. 1). And all these isolate gave positive result in PCR test.

Figure, 1: Colony of *Brucella abortus* on Brucella agar showing the pearly white colony.

Results of PCR (Direct separation of DNA from milk samples) clarified that 32 out of 60 (53.33%) milk samples contain DNA of *Brucella abortus.* Which gave PCR band in size 223 pb as (Fig. 2), and 28 milk sample gave negative result in PCR test with ratio (46.66%). The relation between positive results of rose Bengal test and bacterial culture test revealed to found 53 out of 60 which is gave negative results in Brucella culture method with ratio (88.33%). While the relation between rose Bengal test and PCR test indicated that 28 out of 60 milk samples were negative to PCR test, which is taken from cow that gave positive results in rose Bengal test with ratio (46.6%).



Figure, 2: Electrophoresis on 2 % agarose gel and ethidium bromide staining, showing the results of PCR procedures. M: DNA marker, CP control positive, CN: control negative, wells 1-8 positive samples band in size 223 bp.

The relation between bacterial culture and PCR results**,** revealed that 25 out of 32 (78.12%) milk samples was negative to bacterial culture while its positive to PCR test, and seven milk samples was positive for both bacterial culture and PCR test. This study showed 28 out of 60 (46.66%) milk samples negative to bacterial culture and PCR test (Table, 2).

Table, 2: Compare between bacterial culture and PCR.

	Results of PCR				Total
	Positive		Negative		
Results of culture	Positive	7	Positive	0	7
	Negative	25	Negative	28	53
Total	32		28		60

Brucella are fastidious and relatively slow growing organisms. There were many selective media for the primary isolation of Brucella from grossly contaminated clinical materials, such as milk samples (7). In this study *Brucella abortus* was isolated from 7 out of 60 case which gave positive results to rose Bengal test, Brucella antibody that detected by rose Bengal test maybe from previous infection or from vaccine or due to cross reaction with antibody from other bacterial infection such as Salmonella group, *E. coli* (O:116), *Pseudomonas multophilia*, *vibrio cholera*, *Yersinia enterocolitica* O:9 (10).

PCR is more efficient than culture techniques, because its ability to detect small numbers of bacteria present in the sample even died bacteria (7, 13 and 14). Therefore the treated animals will be detected by PCR, while culture techniques fail to detect such treated animals. Since, 25 (41.7%) of samples were negative by culture technique, while it was positive for *B. abortus* DNA using PCR. Although that gold standard for the diagnosis of brucellosis is isolation of the causative agent (15). There are many factors affect the efficiency of culture techniques like the size of samples, type of culture media, types of the inhibitory additives, number and viability of the bacteria in the samples, and number of samples that taken from the same animal (16). The false-negative bacteriological results may be due to massive contamination of the milk samples or from inhibition of some Brucella spp. by selective medium supplements (8). The consumption of contaminated milk is the main transmission ways to infect humans by Brucella. Therefore, fast and accurate diagnosis of brucellosis status of the milk showed be taken as soon as possible. Because of differences in results of rose Bengal test, Brucella culture method and PCR test in cow milk so the present study suggests that accurate evaluation of brucellosis status of cow milk was the PCR test.

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استعمال اختبار تفاعل انزيم البلمرة المتسلسل في تقييم طريقة الزرع الجرثومي المستعملة في تشخيص البروسيلا المجهضة في حليب الأبقار المشتبه بإصابتها بداء البروسيلات

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الخلاصة

هدفت الدراسة إلى تحديد حساسية الزرع الجرثومي في الكشف عن جراثيم البروسيلا المتواجدة في حليب الأبقار المجهضة ولهذا الغرض جمعت 60 عينة حليب من أبقار مجهضة في حقبة لم تتجاوز شهرين بعد الإجهاض وكانت جميعها إيجابية لاختبار روز بنغال. دلت نتائج البحث على أنّ جراثيم البروسيلا المجهضة عزلت من حليب الابقار بنسبة 11.6% (7 من أصل 60 عينة) في حين كشف اختبار تفاعل انزيم البلمرة المتسلسل عن وجود 32 عزلة عائدة لنمط البروسيلا المجهضة وبنسبة 53.3%. خصوصية الزرع الجرثومي مقارنة مع تقنية تفاعل انزيم البلمرة المتسلسل كانت 10% بينما حساسيته كانت 21.8%. بالإضافة إلى المحاذير في التعامل مع الجراثيم الحية في الزرع الجرثومي، فإن تقنية تفاعل انزيم البلمرة المتسلسل كانت أفضل من الزرع الجرثومي لتشخيص الجرثومة، لذا فإن استعمال تقنية تفاعل انزيم البلمرة المتسلسل أفضل للكشف عن جراثيم البروسيلا المجهضة في حليب الحيوانات المجهضة.

الكلمات المفتاحية: البروسيلا المجهضة، الحليب، تفاعل انزيم البلمرة المتسلسل، الأبقار، داء البروسيلات.