Detection of *Mycobacterium paratuberculosis* in raw cow's milk using polymerase chain reaction (PCR) technique

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Abstract

Paratuberculosis or Johne's disease is a chronic debilitating disease mainly infects ruminants and caused by *Mycobacterium paratuberculosis*. Previous serological studies in Mosul city confirm the presence of positive reactants for paratuberculosis in cattle. However, culture methods to confirm the disease need a long incubation period and also special media. Raw cow's milk is considered as potential source for transmission of *M. paratuberculosis* in cows' herds. Accordingly, this study aimed to detect the presence of *M. paratuberculosis* specifically in the raw cow's milk using polymerase chain reaction (PCR) technique as a rapid, sensitive and reliable method. A total of 50 samples of raw cow's milk were collected from cows suffering from emaciation and unresponsive to antibiotic treatment. All the samples were subjected to DNA extraction and direct amplification PCR. The results showed that 3 (6%) out of 50 milk samples were positive for *M. paratuberculosis*. This is the first study in Mosul city that confirms the presence of *M. paratuberculosis* in raw cow's milk using PCR technique. In conclusion, raw cow's milk could be an important source for *M. paratuberculosis* infection in dairy cows, and also PCR technique could be helpful in rapid diagnosis of paratuberculosis.

الكشف عن العصيات نظيرة السل في حليب الأبقار الخام باستخدام تقنية تفاعل البلمرة المتسلسل

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الخلاصة

يعتبر مرض نظير السل أو مايسمى بمرض جونز من الامراض المزمنة المسببة للهزال والتي تصيب بشكل رئيسي المجترات ويسبب المرض جرثومة العصيات نظيرة السل. اثبتت الدراسات المصلية السابقة في الموصل وجود المرض في الابقار. ومع ذلك فان اثبات وجود المرض باستخدام طريقة العزل الجرثومي تحتاج الى وقت طويل للحضن فضلاً عن اوساط زرعية خاصة. يعتبر الحليب من المصادر المهمة لانتقال العصيات نظيرة السل في قطعان الأبقار. لذلك هدفت هذه الدراسة الى التحري عن وجود عصيات نظيرة السل في حليب الابقار باستخدام تفاعل البلمرة المتسلسل كطريقة سريعة وحساسة وموثوقة. حيث تم جمع ٥٠ عينة حليب خام من الابقار التي تعاني من الهزال وعدم الاستجابة للمضادات الحياتية. خضعت جميع العينات لاستخلاص الدنا ثم تم بعدها إجراء تفاعل البلمرة المتسلسل. أظهرت النتائج وجود ثلاث حالات موجبة للعصيات نظيرة السل وبنسبة ٦% من مجموع العينات البالغة ٥٠ عينة. تعتبر هذه الدراسة الأولى من نوعها في مدينة الموصل في اثبات وجود العصيات نظيرة السل في عينات الحليب الخام للأبقار باستخدام تفاعل البلمرة المتسلسل. يستنتج من هذ الدراسة أن الحليب الخام قد يكون مصدراً مهما لانتقال الاصابة بالعصيات نظيرة السل في أبقار الحليب، كما وان استخدام تقنية تفاعل البلمرة المتسلسل يساعد في التشخيص السريع لمرض نظير السل.

Introduction

Mycobacterium paratuberculosis is the causative agent of paratuberculosis (Johne's disease), a chronic wasting disease, which mainly infects ruminants and can frequently, be found in dairy cattle (1,2). The disease has worldwide distribution and leads to reduce milk yield of the affected herds and contribute to economic loss (2-4). The bacteria are mainly shedding through feces of the infected cattle (5). However, the milk also considers as an important source of infection for the other animals (6). Many studies are confirming the role of raw milk and colostrum as a potential source of infection (6,7). The gold standard method for diagnosis of paratuberculosis is bacterial culture. However, this method is cumbersome and time-consuming, and cultured samples may need up to 3 months to consider negative (7). Therefore, the diagnosis is mainly relied on serological (8-10) and molecular methods (6,7,11,12). Paratuberculosis has been confirmed previously in several studies in Iraq. A previous preliminary study in Mosul city, Iraq confirmed the presence of seropositive antibodies against *M. paratuberculosis* in different ruminant's species including sheep (10), goats (13) and cattle (9) using enzyme-linked immunosorbent assay (ELISA). Another recent study in 2018 by Al-Farwachi and colleagues (14) confirm the presence of antibodies against *M. paratuberculosis by* ELISA in both diarrheic and non-diarrheic or apparently healthy cattle that brought to the Veterinary Teaching Hospital of College of Veterinary Medicine, Mosul University, Mosul, Iraq. Molecular methods offer rapid sensitive techniques for the diagnosis of paratuberculosis. Hence, the use of polymerase chain reaction as a molecular method can help in rapid detection of *M. paratuberculosis* in different types of samples (6,7,15,16), and offer a rapid diagnosis of the disease. Accordingly, this study aimed to detect the presence of *M. paratuberculosis* in raw cows' milk in Mosul city using PCR technique.

Materials and methods

Sample collection

Total of 50 samples of raw cow′s milk was collected from cows suffering from emaciation, unresponsive to antibiotic treatment and retreating at Veterinary Teaching Hospital, College of Veterinary Medicine, University of Mosul, during the period of May to July 2018. A volume of 10 ml of milk was collected in a sterile test tube and transported to the Research Laboratory, Department of Veterinary Public Health, College of Veterinary Medicine. The samples were stored at -20 °C until being used for the DNA extraction process. For the preparation of samples, 10 ml of each milk sample was centrifuged at 3000 ×*g* for 15 min. The supernatant was discarded and the pellet was resuspended in 100 μl of saline solution (17).

DNA extraction

Mycobacterium paratuberculosis DNA was extracted using a commercial DNA extraction kit (*Mycobacterium paratuberculosis* Vet, Ref: VET-36, Sacace Biotechnology, Italy). According to manufacturer instructions, 1.5 ml Eppendorf tubes were prepared according to the number of tested samples. Each tube contains50 μl of milk sample, 20 μl of internal control and 50 μl of negative control of extraction. Additionally, 50 μl of negative control was added to a separate tube as negative control. 300 μl of lysis solution was added to each tube and vortexed and incubated for 5 min at 65 °C. All tubes were centrifuged for 30 sec at 8000 ×*g*. The supernatant was carefully removed without disturbing the pellet. After that, 300 μl of washing solution 1was added to each tube, then vortexed vigorously and centrifuged for 30 sec at 8000 ×*g*. The supernatant was discarded and 500 μl of washing solution 2 was added to each tube and vortexed vigorously and centrifuged for 30 sec at 8000 ×*g*. The supernatant was discarded and the pellet was resuspended in 50 μl of DNA-eluent and incubated for 5 min at 65 °C and vortexed periodically. After that, the tubes were centrifuged for 1 min at a maximum speed of 16000 ×*g*, and the supernatant contains purified DNA was kept at -20 °C for PCR assay.

Amplification and gel electrophoresis

The amplification of the target *M. paratuberculosis* gene was performed according to the manufacturer instruction (Mycobacterium paratuberculosis Vet, Ref: VET-36, Sacace Biotechnology, Italy). Briefly, PCR-mix-1tubes were prepared and 10 μl of PCR-mix-2 was pipetted into each PCR-mix-1 tube. Then, 10 μl of extracted DNA was added to the corresponding tubes. For the negative control tube, 10 μl of DNA buffer was added. Additionally, for positive control tube, 10 μl of positive control (diluted 1:10 with DNA buffer) was added. The PCR was performed using Eppendorf Thermocycler (Eppendorf, Germany) with one cycle at 95 °C for 2 min, 42 cycles consisting of (step 1: 95 °C for 1 min, step 2: 66 °C for 1 min, step 3: 72 °C for 1 min). Then, one cycle at 72 °C for 1 min was set for final extension. Finally, the reactions were cooled at 10°C until proceeding to the gel electrophoresis. Analysis of results was based on the presence of specific bands of amplified gene using 2% agarose gel stained with ethidium bromide (Amersham Bioscience, USA), and visualized using UV transilluminator and digital camera. The presence of specific amplified DNA fragment with209 bp indicating a positive result for *M. paratuberculosis*, in addition, internal control with a product size of 700 bp must also appear to ensure that the extraction was valid.

Results

The results showed that only 3 out of 50 milk samples were positive and the incidence rate was 6%. PCR products with a size of 209 bp were detected in all positive samples. In addition, internal control bands were also detected with product size of 700 bp, indicating successful DNA extraction as shown in Figure (1).
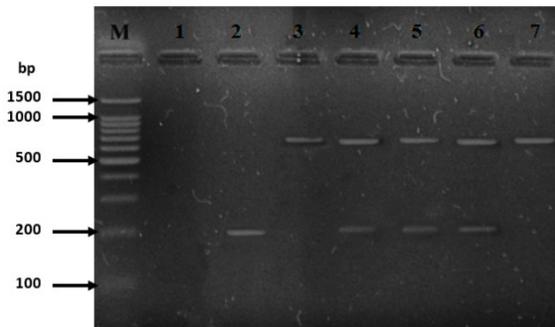

Figure 1: Result of DNA amplification of *M. paratuberculosis* electrophoresed on 2% agarose gel stained with ethidium bromide. Lane M: DNA Ladder, lane 1: negative control, lane 2: positive control, lanes 4, 5 and 6 are positive milk samples with 209 bp product size, lanes 3 and 7 are negative milk samples for *M. paratuberculosis.*

Discussion

Paratuberculosis has gained great importance worldwide due to severe economic losses (3,4). The disease mainly affects ruminants including cattle, sheep and goats, and causes chronic contagious enteritis that characterized by unresponsive to treatment (18,19). Recent studies in Mosul city referred to the presence of seropositive reactant against *M. paratuberculosis* in sheep, goats and cattle which give evidence of the importance of the disease in ruminant (9,10,13,14). In this study, *M. paratuberculosis* was detected in (6%) 3/50 of tested cow's milk, which indicates direct shedding of the pathogen in the raw milk. Since paratuberculosis is a chronic disease and clinical signs may only appear after years of infection, the secretion of *M. paratuberculosis* in milk is considered as main risk factor for transmission and spreading of the disease to calves (6,11,20,21). In addition, the zoonotic potential of *M. paratuberculosis* via consumption of contaminated milk and milk products is of public health importance due to frequent isolation of *M. paratuberculosis* from Crohn's disease patients (22-25). The PCR used in our study offer sensitive and rapid method for detection and confirmation of the presence of *M. paratuberculosis* in clinical samples (6,7,11). Unlike, culture method that requires special media

and experienced personnel, PCR might be conducted in few hours in the equipped laboratory, while culture method might need several months to consider negative results (6,7,25,26). The PCR technique allowed the detection of fewer *M. paratuberculosis* cells in 2 mL of milk, making this procedure is rapid, sensitive and cost-effective for the diagnosis of clinical and subclinical paratuberculosis (11,27). In a study targeting cattle population in south of Iraq 29/81 (35.8%) were positive for *M. paratuberculosis* using PCR from buffy coat of seropositive cows detected by ELISA, and this highlighted the importance of PCR as a confirmatory tool (28). Another study in Brazil, *M. paratuberculosis* was detected in 20 (16.5%) out of 121 of milk samples using conventional PCR (29). Another study in Cyprus confirms the presence of *M. paratuberculosis* in 63 (28.6%) positive samples out of 220 milk samples collected from milk storage (25). However, in our study the lower number of positive samples compared with these studies may be due to sporadic cases that have been received in our Veterinary Teaching Hospital rather than detection of infected farms.

Conclusion

In conclusion, *M. paratuberculosis* was detected successfully in raw cow's milk using PCR which indicates the significance of milk as a potential source of infection in dairy cows.

Acknowledgments

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