DIFFERENTIATION OF EGYPTIAN FIELD ISOLATES FROM VACCINAL STRAINS S19 AND RB51 OF BRUCELLA BY MULTIPLEX POLYMERASE CHAIN REACTION

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ABSTRACT

Brucellosis eradication programme depends on vaccination, test, slaughter and quarantine as control measures. It is essential to distinguish vaccine strains from strains that cause infections among vaccinated cattle herds. Multiplex polymerase chain reaction (PCR) assay was applied to identify and differentiate between the vaccine all strains and field isolates, Two pair primers were used to amplify eri and wbo regions of DNA sequences those strain-specific targets for B. abortus S19 and RB51 vaccinal strains. This multiplex PCR method evaluated with DNA from reference strains, two vaccinal strains and 27 field strains of Brucella isolated from milk of 300 dairy bovine. The results showed that the multiplex PCR can be used to differentiate Brucella isolates into three categories: strain 19 (S19), strain RB51 and field strains. This PCR assay was successfully used, compared with traditional method to differentiate of S19 and RB51 vaccinal strains from field Brucella isolates.

Key wards : *Brucellosis, Multiplex, polymerase chain reaction, field strain, vaccine.*

INTRODUCTION

Bovine brucellosis is a zoonotic disease distributed worldwide and characterized by abortion and reduced fertility in cows. The disease is caused by *Brucella abortus,* which is able to survive and multiply within the tissues of the reticuloendothelial system **(Samartino and Enright, 1993)**. Vaccination with live, attenuated B. abortus strains has been effective in preventing *B. abortus* infection and abortion in cattle. Until recently, strain 19 (S19), a naturally smooth and attenuated strain of *B. abortus*, had been used as a vaccine for cattle brucellosis. There are several disadvantages with S19 vaccination such as cross reactivity in diagnostic tests and abortion in pregnant cattle **(Nagy *et al.,* 1967; and Diaz et al., 1968)**. So vaccinal strain RB51 was developed and vaccination with this strain is as effective as vaccination with S19 in protecting cattle against brucellosis **(Cheville *et al.,* 1993; and Cheville *et al.,* 1996)**. Vaccinal strain RB51 (S-RB51) is an attenuated, rough organism which essentially lacks the O-side chain of the LPS **(Schurig *et al.,* 1991)**. Lack of O-side chain means that this vaccine can be given one or several times without inducing antibodies that interfere with conventional diagnostic tests **(Schurig *et al.,* 1991)**.

Brucella AMOS PCR assay (B**ricker and Halling, 1994)** was developed at the National Animal Disease Center to identify and differentiate *Brucella abortus, B. melitensis, B. ovis,* and *B. suis* bacteria (AMOS is an acronym for the *Brucella* species identified). An abbreviated multiplex AMOS PCR assay was developed to differentiate *B. abortus* into three categories: field strains, vaccinal strain 19 (S19), and vaccinal strain RB51 and the RB51 parental strain, U.S. Department of Agriculture challenge strain 2308 (S2308) **(Bricker and Halling, 1995)**. The abbreviated AMOS assay is based on the insertion of the genetic element IS711 at a unique chromosomal locus in *B. abortus* bv. 1, 2, and 4; and the double insertion of IS711 at a specific locus in *B. abortus* RB51 **(Bricker and Halling, 1994 and 1995)**. One PCR primer is anchored within the IS711 sequence, while the differentiating primers are localized in the unique chromosomal DNAs adjacent to the insertion. Primers were selected to amplify up to three products of different sizes. The primers amplify a 498-bp product present in *B. abortus* bv. 1, 2, and 4 plus two vaccinal strains, and they also amplify a 364-bp product from *B. abortus* RB51.

Identification of S19 is based on a PCR primer pair which amplifies a short sequence (178 bp) **(Bricker and Halling, 1995)** of the eri gene (essential for erythritol catabolism), present in all Brucella strains except *B. abortus* S19 **(Sangari *et al.*, 1994)**. Thus, the identification of S19 is based on the absence of amplification of this target. The classical method of identifying the species and biovars of *Brucella* strains requires a minimum of 5 days. A PCR procedure can differentiate the vaccinal strains from the field strains in 24 h and will provide useful, early information to regulatory officials.

Since the brucellosis eradication programme in Egypt uses vaccination, test, slaughter and quarantine as control measures, it is essential to distinguish vaccinal strains from strains that cause infections in vaccinated cattle herds. Although bacteriological characterization and biotyping process of *Brucella* organismsis were reliable and usually applied to distinguish vaccinal strains, this conventional method has some disadvantages. First, the length of the method from clinical specimen to definitive identification takes time, typically, two weeks. Second, the tests are complex and must be performed in an authorized laboratory by highly-skilled personnel. Some of the characteristics are subjective, such as colony morphology, and require the trained eye and an experienced hand. Third, the zoonotic nature of most Brucella species is a potential hazard for laboratory personnel who must manipulate the infectious agent during testing. Finally, the results are not always definitive **(Bricker, 2002)**. Therefore, a rapid test which is highly sensitive and highly specific is needed more than ever. Hence a multiplex PCR assay used to distinguish S19 and RB51 vaccinal strains from all field strains of Brucella isolated in Egypt. The purpose of this paper is to distinguish between field strains and vaccinal strains S19 and RB51 and evaluate this multiplex PCR as a rapid screening test for field strains and vaccinal strains.

MATERIAL AND METHODS

Samples:

300 bovine milk samples were collected from 200 cattle and 100 buffaloes from different localities at Dakahalia and Damitta provinces. All were subjected for bacteriological examination

Reference strains:

Reference and vaccinal strains (S19 and RB51) were obtained from Veterinary Serum and Vaccine Institute, Abbassia, Cairo, Egypt.

Bacteriological analysis:

Brucella species, biovars and vaccinal strains were identified by morphological, serological and conventional biochemical tests, growth in the presence of thionine, fuchsin, rifampicin, benzylpenicillin and erythritol. Strains were tested by monospecific A, M and R antisera and phage lysis with Tibilissi (Tb) phage according to the standard procedures **(Alton *et al.,* 1988; and Schurig *et al.,* 1991)**.

Genomic DNA extraction:

A medium-sized bacterial colony suspended in 200 µl of sterile distilled water, incubated in a boiling water bath for 15 min, and centrifuged for five min at 10,000 x g, and then two µl of the supernatant was used as template DNA for the polymerase chain reaction (PCR) amplification **(Holmes and Quigley, 1981)**.

Primers:

Two pairs of strain-specific primers were used to show differences between vaccinal strains (S19 and RB51) and field strains of Brucella. Multiplex PCR assay contained the following primers: wbo1 5´- GCC AAC CAA CCC AAA TGC TCA CAA-3´ and wbo3 5´-TTA AGC GCT GAT GCC ATT TCC TTC AC-3´ for RB51 detection (Vemulapalli et al., 1999); eri1 5´- GCG CCG CGA AGA ACT TAT CAA- 3´and eri2 5´-CGC CAT GTT AGC GGC GGT GA-3´ for S19 detection (Bricker and Halling, 1995; and Ewalt and Bricker, 2000). These primers were designed previously based on the following strain-specific genetic differences for two PCR assays separately: a) a wboA gene disruption by an IS711 element in the B. abortus RB51 **(Vemulapalli *et al.,* 1999)**; and b) a 702-bp deletion in the eri operon (essential for erythritol catabolism) in the B. abortus S19 **(Sangari *et al.,* 1994)**.

DNA amplification and detection of PCR product:

PCR amplifications were performed in a 25-µl volume using thermal cycler (MG 5331, Eppendorf, Hamburg). The following PCR conditions were applied to each assay; 50 mM KCl, 10 mM Tris-HCl (pH = 9.0), 2mM MgCl2, 200 µM dNTPs, 10 pM of each primer, 1.25 U *Taq* DNA polymerase (Fermentas) and 2µl of template DNA. After initial denaturation of template DNA at 95°C for seven min, the PCR profile was as follows : 30 cycles of 45s of template denaturation at 95°C; 60 s of primer annealing at 64°C and 45s of primer extension at 72°C; with a final extension at 72°C for six min. The presence of PCR products was determined by electrophoresis of 10 µl of reaction product in a 1.5% agarose gel in TBE (89 mM Tris-HCl, 89 mM boric acid, 2.0 mM EDTA, pH = 8.0) electrophoresis buffer and were visualized by staining with ethidium bromide (0.5 µg/ml) under UV light. Images

were captured on a computer. Sterile water was used as the negative control. At least each sample was tested in duplicate.

RESULTS

Bacteriological examination:

By isolation, Brucella organisms were detected in milk of 27 (9%) out of 300 examined cattle and buffaloes. All isolates were typical of isolates of *B. melitensis* in morphology, colonial appearance and growth characteristics. None of isolates was dependent on CO2 for growth and none of them produced more than traces H_2S.

DNA was successfully extracted from all Brucella reference, vaccinal and field strains. When the two pairs of primers were used together in the reaction, different sizes of fragments were amplified. As expected, wbo1 and wbo2 produced ~1,300-bp fragment from RB51 and ~400-bp fragment from other field strains. Primers eri1 and eri2 amplified a 178-bp fragment from the field strains and RB51 genomic DNA but none from that of S19 strain.

Analysis of representative field isolates also showed agreement with the identifications made by the conventional methodology.

Table (1): Isolation of Brucella from bovine milk samples.

No. of milk samples processed					
Animal	**Tested No.**	**Positive No.**	**%**	**Negative**	**%**
Cattle	200	23	11.5%	177	88.5%
Buffaloes	100	4	4%	96	96%
Total	300	27	9%	273	91%

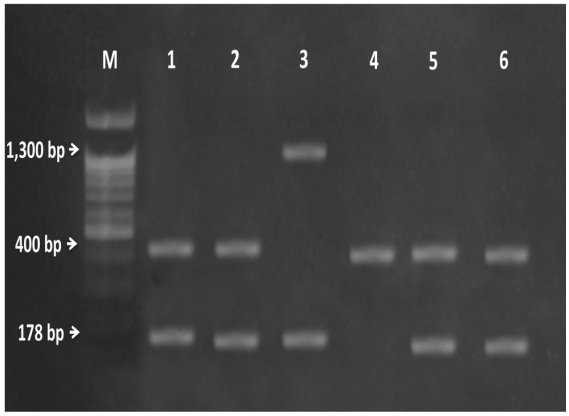

Figure 1: Electrophoretic analysis (1.5% agarose gel) of DNA amplified fragments from different Brucella strains. DNA was amplified by multiplex PCR. M PCR marker Lane 1 and 2: B.abortus strain 554 and B.melitensis H38 ; Lane 3: B. abortus strain RB51; Lane 4: B. abortus strain 19; Lane 5 and 6 field isolates.

DISCUSSION

In brucellosis eradication programs and appropriate action for each epidemiological source of infection, it is necessary to differentiate vaccinal strains from field strains **(Bricker, 2002)**. Accurate typing of Brucella is critical for eradication and control of disease-causing organisms. Classical method of identification of the species and biovars of Brucella strains requires a minimum of five days. Application of molecular assays may usefully provide high sensitivity and specificity as well as speed for genotyping. Multiplex PCR in Brucella was first used in the USA (**Bricker and Halling, 1994)** exploiting the multi-copy element IS711 **(Halling et al., 1993)**, also known as IS6501 **(Ouahrani et al., 1993)**. PCR assay was named "AMOS" which is an acronym for the Brucella species where it can identify. AMOSPCR can identify and differentiate B. abortus, B. melitensis, B. ovis and B. suis. As more Brucella gene sequences were published **(Sangari et al., 1994; and Vemulapalli et al., 1999)**. This PCR assay was modified to distinguish the two commonly used vaccinal strains: S19 and RB51 among wild field strain by strain-specific primers **(Bricker and Halling, 1995; and Ewalt and Bricker, 2000)**. Use of the modified AMOS technique **(Bricker and Halling, 1995)**, in Iran, for diagnostic purposes is not suitable (**Sharifi et al., 2008)**, because of the difference between the Brucella species in Iran country and strains present in the USA. For instance, B. abortus biovar 3 which is the most frequent biovar existing in Iran **(Zowghi and Ebadi, 1982)** cannot be detected by this method **(Bricker and Halling, 1994; and Adone et al., 2001)**. In addition, unresolved bands ranging from 600- to 700-bp were reported from RB51 in this method **(Adone et al., 2001)**. They developed a multiplex PCR assay that can identify and differentiate two vaccinal strains from field isolates and biovars. Originally, PCR assay that distinguished RB51 from other strains was performed with combinations of primers wbo1, wbo2 and wbo3, whereas in multiplex PCR only the reaction mixtures contained wbo1 and wbo3 primers with two additional primers of eri1 and eri2 from modified AMOS-PCR to distinguish RB51 and S16 vaccinal strains from other strains **(Bricker and Halling, 1995; Vemulapalli et al., 1999 and Ewalt and Bricker, 2000)**. These four primers were employed in a multiplex PCR to amplify eri and wbo regions of DNA sequences of those vaccinal strain-specific targets for S19 and RB51 respectively and produced PCR amplified fragments of different sizes which make it easy for analysis on agarose gel. These primers were chosen to allow a more distribution of amplicon sizes for easier analysis. In addition, combination of these primers allowed an internal control for S19 strain that producing a 400-bp fragment in this multiplex PCR assay. Field strains of Brucella amplified two DNA fragments of 178- and 400-bp, while the oligonucleotide primers, allowed the amplification of 178-bp plus a 1300-bp fragment specific for RB51. In addition, the identification of S19 is based on the presence of a 400-bp fragment as the only product and the absence of amplification of a 178-bp fragment. So, identification of S19 is based on a PCR primer pair which amplifies a short sequence (178-bp) of the eri gene, present in all Brucella strains except B. abortus S19 **(Sangari et al., 1994; and Bricker and Halling, 1995)**. In a study which employed four-pair primers to differentiate all Brucella species and vaccinal strains, these two regions of gene (eri and wbo) were also used to distinguish vaccinal strains **(Garcia-Yoldi et al., 2006)**. Based on these results, PCR assay with primers wbo1, wbo3, eri1 and eri2 is recommended to distinguish strain RB51 and S19 from all other Brucel-

la strains. The assay described in this report has several advantages over the current microbiological methods used to identify Brucella species. A major advantage is the speed with which the assay can be performed i.e. within less than one working day, this method provides useful and early information to make a decision. In addition, the bacteria can be killed and sent for identification. This is very important because Brucella is a human pathogen. Comparison of the conventional methods and multiplex PCR results for the diagnosis of B. abortus vaccine or Brucella field strains, showed 100% agreement between both tests. Samples were correctly identified as either Brucella field strain; B. abortus vaccinal strain RB51 or B. abortus vaccinal strain S19. This PCR assay has provided methods for the direct analysis of cultured bacterial samples. In future studies, this PCR method can be used as a direct test for differentiation of post-vaccine infection from field infection in clinical samples such as tissues, semen, milk and blood of cattle. However, it is necessary to remove PCR inhibiting components and needs extensive sample preparation (L**eal-Klevezas et al., 1995).**

The main advantage of the assay is its ability to differentiate two vaccinal strains which are used in Iran from field isolates. This PCR is rapid, very specific and easily adapted to high volume demands. As long as careful attention is given to avoid contamination, the method is very reliable and usually highly reproducible at any properly equipped laboratory so this assay can be used by National Veterinary Services Laboratories, where most of the suspected veterinary samples are submitted for identification of causative organisms. This multiplex PCR assay has the ability to quickly verify the presence or absence of RB51 and S19 among Brucella isolates from clinical samples

CONCLUSION

In this study, PCR was shown to be a valuable tool for differentiating vaccinal strains from field strains of Brucella. Conventional methods of identification require a minimum of 5 days to identify an isolate of Brucella species at biovar level. This can delay the movement of cattle between different owners and have a negative impact on the owners' financial planning. This study indicates that Brucellosis eradication program personnel could reliably use the abbreviated Brucella AMOS PCR to supplement other diagnostic and epidemiological data (such as herd history and serological test results) to release sale animals from quarantine before the conventional identification methods are completed.

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الملخص العربى

التفرقة بين العترات الحقلية والعترات المستخدمة فى التحصين **S19** و **RB51** لمرض البروسيلا باستخدام تفاعل البلمرة المتسلسل المتعدد بمصر

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برنامج القضاء على مرض البروسيلا فى مصر يعتمد على التحصين والاختيار الدورى للحيوانات وذبح الحيوانات المصابة وعمل حجز بيطرى للحد من انتشار المرض لذا كان من الضرورى التفرقة بين العترات الحقلية والعترات المستخدمة فى التحصين فى الحيوانات المحصنة فى هذه الدراسة استخدام تفاعل البلمرة المتسلسل المتعدد للتعرف والتفرقة على العترات المعزولة إذا كانت عترات حقلية أو عترات استخدام فى تحصين الحيوانات، وفى هذا التفاعل تم استخدام زوجين من البرايمرات التى أستخدم فى مضاعفة الحامض النووى وهما (eri,wbo) wbo أستخدم للكشف عن العتره المستخدمة فى تحصين RB51 أما eri استخدم للكشف عن العترة المستخدمة فى تحصين S19. وتم تقيم هذا التفاعل باستخدام 27 عترة معزولة من إجمالى 300 عينة لبن مجمعة من 200 من الماشية و 100 من الجاموس بعد عزلها والتعرف عليها بالطرق التقليدية ومقارنتها بعترات التحصين، وأوضحت النتائج أن كل العترات المعزولة كانت من العترات الحقلية ولم يتم عزل أى عترات مستخدمة فى التحصين سواء RB51 أو 519 وقد تبين أن هذا التفاعل له القدرة على التفرقة بين العترات المغزولة بطريقة دقيقة وأسرع من الطرق التقليدية التى قد تتطلب أكثر من 5 أيام للتعرف على المعزولات.

الكلمات الدالة :

Brucellosis, Multiplex, polymerase chain reaction, field strain, vaccine.