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DIFFERENTIATING BRUCELLA MELITENSIS VACCINE STRAIN REV. I AND FIELD STRAINS USING POLYMERASE CHAIN REACTION

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Distinguishing vaccine strain from field strains is essential for epidemiological investigation of the B. melitensis infection.The objective of this study was to develop strain specific primers and configure a polymerase chain reaction (PCR) assay for differentiating B.melitensis from vaccine strain Rev. 1.An anchor-PCR based on a combination of primers one anneals to the IS711 element while the second primer chosen arbitrarily and a random amplified polymorphic DNA PCR (RAPD-PCR) assays were used to generate fingerprints of B. melitensis and Rev. 1. The products of each reaction was then resolved by polyacrylamid gel electrophoresis, unique bands present in each replicates were reamplified, purified, cloned and sequenced. Primers were chosen using computer program (oligo 4.0). As a preliminary result two set of the designed primers gave 300 and 325 bp bands unique to Rev. 1 strain. These primers could be used either in AMOS-PCR (defined by Bricker B.J., Halling S.M., Clin.Microbiol., 33(6),1996) or in separate PCR. We are in the process of testing other potential primers and optimization of PCR.

INTRODUCTION

Brucellosis in sheep and goats caused by *B. melitensis* is still widespread and an economically important zoonotic disease. Research in developed countries has mainly focused on bovine brucellosis which has been reduced or eliminated in several countries. Sheep and goats, however, may infect cattle with *B. melitensis* when they come in contact. Of the four species of Brucella, *B. melitensis* is the most pathogenic to humans. Prevention of brucellosis in humans depends on the control or eradication of the disease in animals. For several reasons, it is not usually possible to conduct a test and slaughter policy in sheep and goats; therefore vaccination has been the primary control strategy of *B. melitensis* infection. Since its first isolation in 1957 *B. melitensis Rev.1* has become widely accepted as an efficient live vaccine. Distinguishing the vaccine strain from field strains is essential for epidemiological investigation of the disease. The objective of this study was to develop strain specific primers and configure a Polymerase Chain Reaction (PCR) test for differentiating *B. melitensis* from vaccine strain *Rev.1*. For this purpose, we attempted to use Anchor-PCR and Random amplified polymorphic DNA- polymerase chain reaction (RAPD-PCR). Anchor-PCR used in this study is based on a combination of primers one bound on the IS711 sequence and the second chosen arbitrarily. The RAPD-PCR utilizes short single primers with randomly chosen sequences to amplify multiple DNA segments without requiring prior DNA sequence information. Produced band patterns (fingerprints) are used to reveal polymorphisms among microorganisms.

MATERIALS AND METHODS

Bacterial strains : The reference strains of *B. abortus 544,B.melitensis M16* and *B. melitensis Rev.1* were supplied by The USDA: Agriculture Research Services - National Animal Disease Center, in Ames, Iowa. The organisms were killed by the addition of 67% methanol and 33% saline and were then rinsed two times in distilled water to remove the methanol, then re-suspended in distilled water at an optical density of .20 at 600nm (approximately 10^9 cells per ml). PCR was performed directly on killed bacteria.

Primers : The oligonucleotite primers used were: primers FO1, FO11, F13 (Kit F Operon, Inc. .Alameda, CA); ten OPA primers (Operon, Inc. Kit A) and *B.abortus, B.melitensis*, IS711 primers (Genosys, Inc. The Woodlands, TX).

Polymerase Chain Reaction (PCR) Assays :

Anchor PCR : The PCR was performed briefly in the final volume of 25 ul containing 1X buffer (Invitrogen, San Diego, CA) which includes 60mM Tris-HCl (pH 9.0), 15 mM $(NH_4)_2SO_4$,1.5 mM $MgCl_2$; 250 uM each of the deoxynucleoside triphosphate (dATP, dCTP, dGTP, dTTP) (Invitrogen San Diego, CA); 0.5uM of the arbitrary primers and 1uM of IS711 specific primer; 1.50 ul TaqStart antibody (Clontech, Inc. Palo Alto, CA) including 1.25 U of taq polymerase (Promega). The reaction mixture was prepared as a master mix in one tube then dispensed into .65ul vol- tubes (22.5 ul per tube) . The mixture was overlaid with 20 ul of mineral oil and a 2.5 ul template was added. The PCR was carried out in an MJ Research Thermal Cycler. Following the hot start, 7.5 min at 95°C, the samples were cycled 35 times: 1.30 min of denaturation at 95°C, 2 min of annealing at 54.5°C,and 2 min extention at 72°C. The annealing time was increased by 4 min in the first 5 cycles and the last extention time was 3 min. For analyses, 4 ul of the PCR product was electrophored in 1.5% agarose (Gibco) gel, stained with ethidium bromide (Sigma), and observed with UV. The sizes of the product were estimated by comparison to a standard 100bp DNA marker included in each electrophoretic run.

RAPD-PCR : This PCR was performed in the final volume of 25 ul consisting of 1X taq-DNA polymerase buffer (Promega, Madison, WI) which includes 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 0.1% Triton X-100; .1% gelatin, 10 mM $MgCl_2$ (Promega), 10 mM dNTPs (250mM each of the deoxynucleoside), 1 pM primer and 1.50ul TaqStart-antibody including 1.5 U of taq polimerase (Promega). The mixture was overlaid with 20ul of mineral oil and a 2.5 ul template (10^7 whole cell for *B. abortus* and *B. melitensis* and 10^6 for Rev.1 per 25ul reaction) was added. Following the hot start, 5 min at 95°C,the samples were cycled 44 times: 1 min of denaturation at 92°C, 1 min of annealing at 35°C, and extention at 72°C for 2 min. Extention time for the last cycle was increased to 7 min. Samples were analyzed as described above.The above two assays were compared to a previously described a Brucella assay (Bricker and Halling) which can distinguish selected biovars of four species of Brucella: B. abortus, B. melitensis, B. ovis, B. suis. This described assay is called "AMOS" on the basis of the initial species that it identifies.

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