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Full Length Research Paper

A new protocol for the treatment of *Brucella melitensis* in Neumann's gazelle (*Gazella erlangeri*) from Saudi Arabia using oxytetracycline and streptomycin

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The presence of *Brucella melitensis* is demonstrated for the first time in Neumann's gazelle (*Gazella erlangeri*). Seven Neumann's gazelles exhibiting signs of brucellosis were treated for one month with oxytetracycline (25 mg/kg) administered intramuscularly in combination with streptomycin (20 mg/kg) for two weeks and then solely with oxytetracycline for a further two weeks. Prior to treatment, both serological and bacteriological tests were positive for *Brucella melitensis*. Specific identification of this zoonotic bacterium was confirmed by polymerase chain reaction (PCR). The primers used in the PCR were those that amplify 31 kDa membrane protein and partial 16S rRNA regions as genus specific primers whereas primers which amplify IS711 region were employed as *Brucella* specific primers. The haematological and biochemical parameters of blood samples taken from the infected gazelles prior to and following the treatment were measured. The findings of this study reveal that long term treatment of the Neumann's gazelles infected with *Brucella melitensis* using a combination of oxytetracycline and streptomycin, followed by only oxytetracycline succeeded in eradicating the infection.

Key words: *Brucella melitensis*, treatment, Neumann's gazelle, oxytetracycline, streptomycin, Saudi Arabia.

INTRODUCTION

Brucellosis is the most common zoonosis in the world, accounting for an annual occurrence of more than 500,000 cases caused by a number of *Brucella* species (Pappas et al., 2006). Human brucellosis in Saudi Arabia has been known since the 1950's but it was not until 1983 that the disease was officially recorded in humans (Kambal et al., 1983) and domestic animals (Radwan et al., 1983, 1989, 1992, 1993; Ramadan et al., 1998). The disease has also been found in a wide range of domestic animals in Qatar (Hamdy and Amin, 2002). Both *Brucella melitensis* and *Brucella abortus* were isolated from blood cultures of human patients and animals with the former less frequently isolated from animals (Abbas and Agab, 2002; Kambal et al., 1983). However, *B. abortus* is believed to occur in the dromedary camel (*Camelus dromedarius*) in Saudi Arabia (Alshaikh et al., 2007a; b). During the past two decades, numerous studies have been published which show that brucellosis is widely distributed across Saudi Arabia and that its incidence is rising progressively in both man and animals. It is currently estimated that the incidence in annual brucellosis is 214.4 per million in the Saudi population (Rust, 2010). The massive expansion of husbandry and importation of unvaccinated and untested livestock resulted in 20% of Saudi Arabians demonstrating seropositivity for brucellosis, while 2% of the population are believed to have active disease. The incidence of

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new cases is highest during the Hajj; the increase is due to numerous cases being recorded among pilgrims to Makkah (Rust, 2010).

One of the main reasons for this unusually high prevalence of human brucellosis is believed to be the consumption of raw camel milk, a long-standing tradition among inhabitants of the Arabian Peninsula.

There is no record of *Brucella* species from wildlife in Saudi Arabia apart from that of Ostowroski et al. (2002) who reported *B. melitensis* in the Arabian oryx (*Oryx leucoryx*) at the National Wildlife Research Centre (NWRC). *Brucella melitensis* has also been detected in a herd of sand gazelle (*Gazella subgutturosa marica*) in the northern regions of Saudi Arabia (Mohammed, Unpublished data).

Several treatment trials for brucellosis have been previously attempted, but none was entirely successful (Gwatkin and Macleod, 1938; Hall and Manion, 1970). Some trials involving small doses of tetracycline injected either alone or in combination with streptomycin (Nicoletti et al., 1985) have also been unsuccessful in treating bovine brucellosis.

The only known captive populations of Neumann's gazelle in the world exist at King Khalid Wildlife Research Centre, Thumamah, Saudi Arabia and Al Wabra Wildlife Preservation close to the town of Al Shahaniyah in central Qatar and kept for breeding and possible later reintroduction purposes in the wild.

This study was undertaken to investigate the occurrence of *B. melitensis* in a herd of Neumann's gazelle (*Gazella erlangeri*) and to evaluate a potential treatment to eradicate zoonosis using a combination of the antibiotics oxytetracycline and streptomycin. The polymerase chain reaction (PCR) was employed for molecular characterization and to evaluate the presence of the *Brucella* organism after treatment.

MATERIALS AND METHODS

Blood samples from 45 Neumann's gazelles, including seven which exhibited enlargement of the carpal joints, were screened for brucellosis. The animals were kept in breeding pens at the King Khalid Wildlife Research Centre, (KKWRC) in Riyadh, Saudi Arabia. Blood samples were collected from each gazelle by jugular venipuncture using 10 ml vacutainers with and without anticoagulant (Becton Dickinson, Vacutainers systems, Rutherford, New Jersey, USA). Serum samples were separated from clotted blood for serological detection of *Brucella* agglutinin using the Rose Bengal test (BioMérieux sa F-69280 Marcy l'Etoile, France).

For bacterial isolation, synovial fluid and anticoagulated blood from infected gazelles and fetal stomach contents from an aborted fetus were inoculated in 5% *Brucella* blood agar (Saudi Prepared Media Laboratories, SPML, and Saudi Arabia) in CO_2 incubator (5% CO_2). The fetal stomach contents were obtained from the fetus of a treated gazelle which was aborted during the treatment period.

DNA was extracted from the isolated organism and from infected gazelle's blood using Qiagen tissue extraction kit (Qiagen, GmbH, Hilden, Germany). The PCR was performed using three sets of primers (MWG-Biotech, Germany). The first set amplifies a 223 bp fragment which is part of the 31KDa membrane protein specific to *Brucella* spp. (Al-Attas et al., 2000; Navarro et al., 2004). The primers used were B4 and B5 with the sequences: B4 5' TGG CTC TGC CAA AAT CAA 3' as forward primer and B5 5' CGC GCT TGC CTT TCA GGT CTG 3' as the reverse primer.

The second pair (F4 and R2) amplifies a 905 bp fragment of the 16S rRNA sequence of *Brucella* spp (Romero et al., 1995). The sequences of the primers used were F4 5' TCA AGC GCC CGC AAG GGG 3' as a forward primer and R2 5' AAC CAT AGT GTC TCC ACT AA 3' as the reverse primer.

The third set amplifies the locus IS711 (previously known as IS6501) which is a species specific locus (Bricker and Halling, 1994) where the sequences for the primers are 2976616-F melitensis 5' AAATCGCGTCCTTGCTGGTCTGA 3' and 2976615-F abortus 5'GACGAACGGAATTTTTCCAATCC 3' as forward primers for *Brucella melitensis* and *Brucella abortus,* respectively, and IS711-R 5' TGCCGATCACTTAAGGGCCTTCAT 3' as the reverse primer for both bacteria.

The PCR was carried out under the following conditions: purified genomic DNA extracted was added to a reaction mixture containing 16 mM $(NH_4)SO_4$, 67 mM Tris-HCl (pH 8.8 at 25°C), 1.5 mM $MgCl_2$, 1 µM of each primer and 200 µM of each deoxynucleotide. Taq DNA polymerase (0.5 units, Advanced Biotechnologies, UK) was added to the reaction mixture when the temperature reached 94°C. The total volume of the reaction was 25 µl. The cycling conditions were as follows: denaturation at 92°C for 30 min, annealing at 54°C to 60°C depending on the primers used for 30 min and extension at 72°C for 30 min for 40 cycles. The PCR product was checked by agarose gel electrophoresis using 1% agarose (Bioline, UK) in 0.5X Tris Borate EDTA (TBE) buffer. The gel was stained using ethidium bromide (0.5 µg/ml), visualized using UV light and photographed using a gel documentation system (Syngene, UK).

Anticoagulated blood samples in EDTA were collected for hematological investigation using Vetscan HM3 (Abaxis, USA) for measuring red blood cells (RBCs), white blood cells (WBCs), and lymphocytes, monocytes, granulocytes, packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelets and platelet indices. Serum samples collected from infected gazelles were used for biochemical investigations using an automated biochemical analyzer VetScan VS2 (Abaxis, USA). Tests performed included albumin (ALB), alkaline phosphatase (ALP), alanine tarnaminase (ALT), amylase (AMY), total bilirubin (TBIL), blood urea nitrogen (BUN), calcium (CA), phosphorus (PHOS), creatinine (CREA), glucose (GLU), sodium (NA), potassium (K), total protein (TP) and globulin (GLOB) tests.

Infected gazelles were housed individually, handled manually and injected intramuscularly every other day with oxytetracycline (Pfizer, Terramycin LA 200 mg/ml) at a dose rate of 25 mg/kg combined with streptomycin (Devomycin, Norbrook Laboratories Ltd., N.Ireland, 250 mg/ml) at a dose rate of 20 mg/kg for two weeks and oxytetracycline at the same dosage but without streptomycin for a further two weeks. Coagulated and uncoagulated blood samples were taken from each individual prior to commencement of the treatment and one month later at the conclusion of the treatment.

Mean hematological and biochemical values were compared using Mann–Whitney rank sum test (Sokal and Rohlf, 1981) using the computer program Sigmastat (Sigmastat Statistical Software, version 2.03, SPSS Inc.). A value of $p \leq 0.05$ was considered to be significant for statistical tests.

RESULTS

Serological, microbiological and molecular investigations indicated that the Neumann's gazelles at KKWRC were naturally infected with *Brucella melitensis. Brucella*

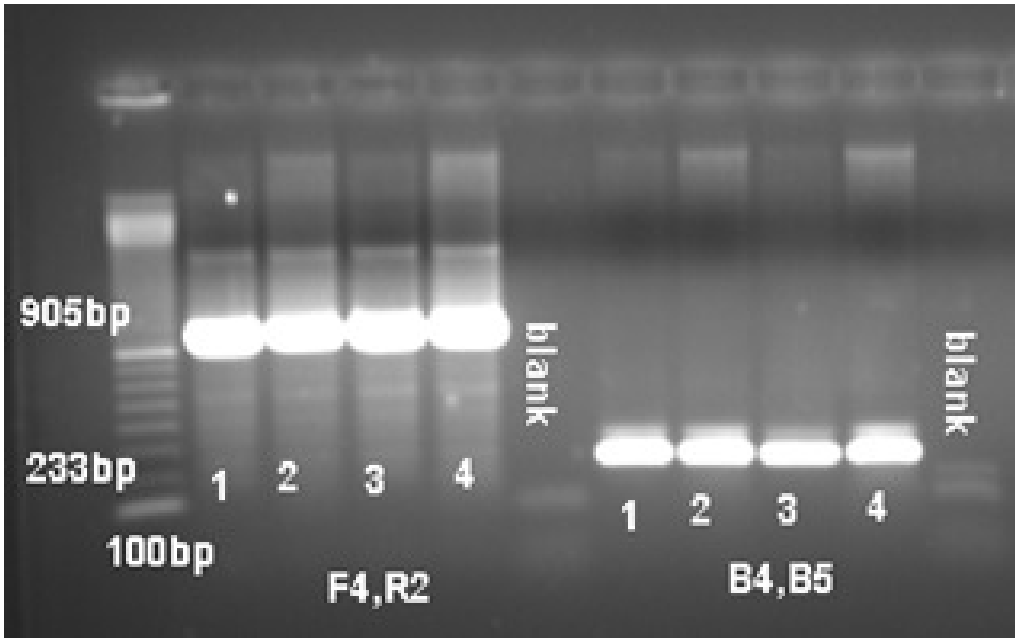

Figure 1. Agarose gel electrophoresis showing PCR product that resulted from the use of primers that amplify the 31-kDa membrane protein (B4 and B5 producing 233bp) and part of 16S Rrna (F2 and R2 producing 905bp) regions. Lanes labelled 1 to 4 shows the samples from Nenmann's gazelles for each primer pairs and the DNA size standard (100bp ladder).

antibodies using the Rose Bengal test; was detected in seven (15.6%) out of 45 gazelles screened in this study. The positive gazelles were those showing swelling of the carpal joint, a clinical sign of brucellosis.

Brucella sp. was isolated from the blood as well as the synovial fluids of the seven infected gazelles but not from the fetal stomach contents of the aborted fetus. The organism isolated from the *Brucella* agar was Gram negative coccobacillary, oxidase and catalase positive, hydrolyses urea, lactose and sucrose non fermenting and H_2S negative, which is suggestive of *Brucella*. No organism was isolated from the blood collected from the remaining 38 gazelles. The DNA extracted from the isolated organism following PCR using specific primers for the genus *Brucella* and for differentiating between *B. abortus* and *B. melitensis* confirmed the identity of the bacterium involved as *Brucella melitensis*. The expected size of PCR product (for both 31 kDa membrane protein and 16S rRNA regions) of DNA extracted from the bacteria isolated from the culture of blood from the seven infected gazelles before the start of the treatment confirmed that the animals were infected with a *Brucella* sp. (Figure 1). Furthermore, the PCR products recovered from the same samples using *Brucella* specific primers that amplify IS711 (731bp) region demonstrated the expected PCR product size to be *B. melitensis* (Figure 2).

Microbiological and molecular results obtained from blood samples collected from the infected gazelles treated against brucellosis for one month were all negative for *Brucella* spp.

Hematological and biochemical results obtained prior to and after the treatment of infected gazelles are tabulated in Tables 1 and 2 respectively. There was a significant increase in hemoglobin concentration ($p<0.05$) in the gazelles following the treatment while the RBC count showed non significant increase ($p>0.05$). There were no significant changes in any of the other hematological parameters. There was a significant increase in the activity of alanine transaminase ($p<0.01$) but a significant decrease in glucose concentration ($p<0.03$) following the treatment. Other biochemical para-meters showed no significant changes.

Four months post treatment, one treated gazelle exhibited enlargement of joints and the results from synovial fluid culture as well as the PCR product from DNA extracted from the synovial fluid proved positive for *Brucella melitensis*.

DISCUSSION

This is the first record of the isolation of *Brucella melitensis* in Neumann's gazelle (*Gazella erlangeri*) and with an occurrence of 15.6% in the herd. Treatment of infected gazelles with a combination of the two antibiotics oxytetracycline and streptomycin for a period of one month proved successful in eliminating the *Brucella* organism from the blood, although abortion occurred in one gazelle four months after treatment. The use of molecular techniques in combination with routine

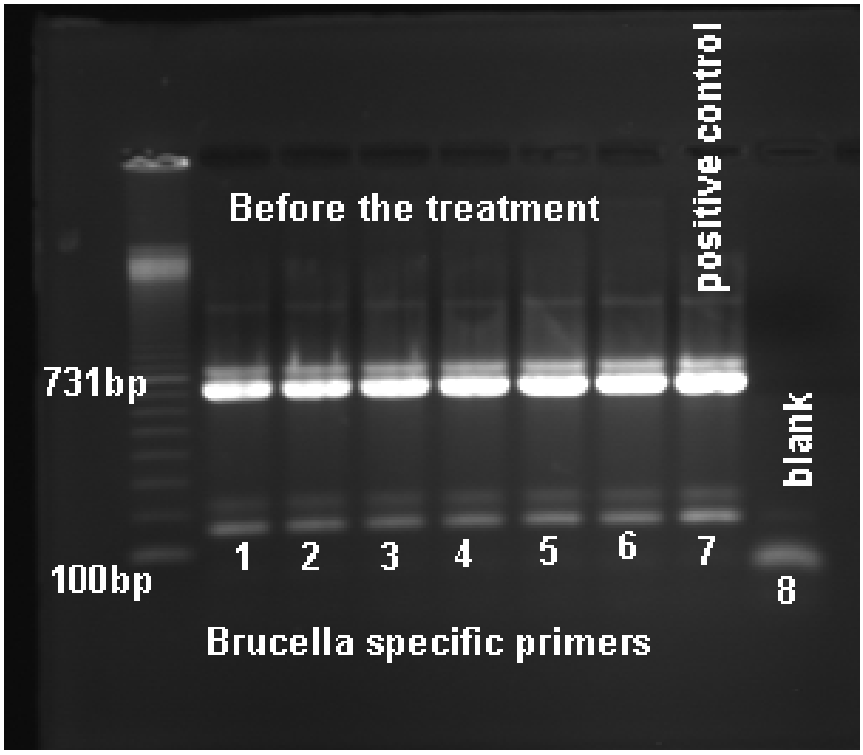

Figure 2. Agarose gel electrophoresis showing the result of using *Brucella* specific primer (2976615-f melitensis/ 2976616-f abortus and 15711-r) from six blood samples (lanes 1 to 6) collected from Neumann's gazelles before the treatment. The first lane was for the DNA ladder (100bp ladder). The fragment size generated corresponds to the expected size (731bp) for *Brucella melitesnis* and not *Brucella abortus* (498bp).

Table 1. Hematological analyses of Nenmann's gazelles infected with *Brucella melitensis* before and after the treatment.

Parameter	Before treatment	After treatment
WBC (10^9/L)	4.6 ± 1.1	5.6 ± 1.9 $^{N.S}$
Lymphocytes (10^9/L)	2.7 ± 0.5	2.9 ± 0.8 $^{N.S}$
Monocytes (10^9/L)	0.3 ± 0.3	0.2 ± 0.2 $^{N.S}$
Granulocytes (10^9/L)	2.6 ± 1.8	1.5 ± 1.0 $^{N.S}$
Lymphocytes (%)	51.3 ± 15.2	64.3 ± 15.7 $^{N.S}$
Monocytes (%)	42.7 ± 16.8	31.4 ± 16.8 $^{N.S}$
Granulocytes (%)	6 ± 3.8	4.3 ± 3.2 $^{N.S}$
RBC (10^{12}/L)	12.6 ± 0.3	13.4 ± 0.8 $^{N.S}$
Haemoglobin (g/dl)	16.1 ± 0.8	17.2 ± 0.7*
PCV (%)	47.9 ± 2.8	50.9 ± 2.8 $^{N.S}$
MCV (fl)	38 ± 2.1	38.6 ± 1.2 $^{N.S}$
MCH (pg)	12.8 ± 0.5	13.1 ± 0.6 $^{N.S}$
MCHC (10/dl)	33.7 ± 0.6	33.8 ± 0.8 $^{N.S}$
RDWC (%)	21.2 ± 3.3	22.3 ± 4 $^{N.S}$
PLT (10^9/L)	269 ± 180.2	413.7 ± 342 $^{N.S}$
PCT (%)	0.2 ± 0.2	0.32 ± 0.3 $^{N.S}$
MPV (fl)	7.2 ± 0.8	7.5 ± 0.9 $^{N.S}$
PDWC (%)	33.2 ± 3	33.9 ± 2.2 $^{N.S}$

$^{N.S}$ No significant difference in values prior and post treatment.
*Indicates significant difference ($p < 0.05$).

Table 2. Biochemical analyses of serum samples collected from Nenmann's gazelles infected with *Brucella melitensis* before and after the treatment.

Parameter	Before treatment	After treatment
ALB (g/L)	12.9 ± 5.9	17.4 ± 2.3 $^{N.S}$
ALP (U/L)	55.9 ± 11.9	68.8 ± 14.2 $^{N.S}$
ALT (U/L)	19.1 ± 4.7	41.1 ± 10.5*
AMY (U/L)	98.9 ± 28.5	108.9 ± 47.4 $^{N.S}$
TRIL (µmol/L)	3.6 ±0.9	3.0 ± 0.1 $^{N.S}$
BUN (mmol/L)	13.1 ± 7.3	9.1 ± 2.6 $^{N.S}$
Ca (mmol/L)	2.3 ± 0.2	2.4 ± 0.1 $^{N.S}$
Phos (mmol/L)	1.8 ± 0.6	1.5 ± 0.4 $^{N.S}$
Creat (µmol/L)	90.1 ± 35.8	81.9 ± 10.1 $^{N.S}$
Glu (mmol/L)	7.8 ± 1.9	5.5 ± 1.6*
Na (mmol/L)	151.7 ± 2.5	152.3 ± 2.9 $^{N.S}$
K (mmol/L)	5.5 ± 0.5	5.6 ± 0.4 $^{N.S}$
Total protein (g/L)	76 ± 11.4	72 ± 6.9 $^{N.S}$
Globulin (g/L)	62.4 ± 13.9	54.6 ± 7.9

$^{N.S}$ No significant difference in values prior and post treatment.
*Indicates significant difference (p$\Re$ 0.05).

bacteriological techniques provided a valuable tool for the identification and subsequent treatment monitoring processes. Long acting oxytetracycline and streptomycin are capable of penetrating the bacterial cell wall, inhibiting protein synthesis and providing long lasting concentrations in the plasma that are considered most effective in the treatment of most animals and humans, since successful treatment depends on the permeability of the cell wall to the drug (Pivnyak, 1958). There is a synergistic effect of oxytetracycline and streptomycin which has been demonstrated both *in vivo* and *in vitro* (Richardson and Holt, 1962).

Several treatment trials of brucellosis have been previously undertaken using penicillin, sulphonamides, phenol and vitamins but all of these trials have been unsuccessful (Hall and Manion, 1970). The *Brucella* bacterium is protected from antibiotics since it survives within phagocytic cells of the reticuloendothelial system. In our investigation, long term treatment of Neumann's gazelles using high doses of the antibiotics oxytetracycline and streptomycin resulted in eradication of the infection in the gazelles as evidenced from both molecular and bacteriological investigations.

Apart from the significant increase in hemoglobin concentration and ALT activity and a decrease in glucose level of the treated animals, there were no significant changes in the hematological and biochemical parameters. Increase in liver enzymes (SD, ALT and AST) was reported previously from camels infected with *B. melitensis* and *B. abortus* (El-Boshy et al., 2009). These authors attributed high levels of liver enzymes and hypoglycaemia to liver damage during infection, while in this study the liver may have been only slightly affected by the infection.

The female which showed enlarged joints aborted four months after undergoing treatment; however, since no *Brucella* organism was isolated from the aborted fetus while PCR results were negative in this female, it appears that the abortion might have been due to a cause other than brucellosis. It has been observed earlier that brucellosis in reem gazelles (*Gazella subgutturosa marica*) was not associated with abortion and that many gazelles infected with *B. melitensis* have given birth to healthy neonates (Mohammed, unpublished data). On the other hand, abortion in the present gazelle could have been due to the use of oxytetracycline, since earlier work at King Khalid Wildlife Research Centre (KKWRC) revealed that pregnant gazelles treated with oxytetracycline aborted during the treatment period (Sandouka, King Khalid Wildlife Research Centre, pers. comm., 2009).

Relapse rate has been frequently studied in the evaluation of treatment protocols for brucellosis (Hajia et al., 2009). Al-Eissa (1999) attributed unpredictable relapses following treatment in humans to an inappropriate choice, dosage, or length of antimicrobial therapy, and even failure of patients to comply with the prescribed treatment. Hajia et al. (2009) reported a relapse rate of 4.78% in 269 patients treated against brucellosis with conrtimoxasole-rifampicin combination. In this study, however, the relapse was certainly not attributable to the improper use of antibiotic or the failure to take the antibiotic indicated for treatment. Moreover, it could be related to the pharmacodynamics of the drugs used for the treatment of brucellosis in the Neumann's gazelle. Antibiotic-resistant *Brucella* strains are rarely a cause of therapy failure and this drug combination has been used previously in Saudi Arabia for the treatment of

brucellosis in sheep caused by *Brucella melitensis* (Radwan et al., 1989, 1992). Hence, the institution of another combination of antibiotics for a longer duration appears warranted to improve the outcome and prevent relapse in Neumann's gazelle.

Thess findings may assist in treating other gazelle species that become infected and therefore, aids in the conservation of Neumann's gazelle and other endangered antelope species in the Kingdom of Saudi Arabia.

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