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LABORATORY INVESTIGATIONS AFTER EYE DROP IMMUNISATION OF DROMEDARIES WITH LIVE ATTENUATED *Brucella melitensis* REV 1 VACCINE

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ABSTRACT

The present study describes the laboratory investigations after a single right eye drop (3.1×10^9 CFU live bacteria) immunisation of 6 dromedary camels (*Camelus dromedarius*) with live attenuated *B. melitensis* Rev 1 vaccine. The experiment was conducted over a period of 5 months. The vaccine strain was isolated for 16 days from only the right eye of the vaccinated dromedaries, but not from the left eye and both nostrils. Similar pattern of results was obtained by polymerase chain reaction. It was negative for the left eye, both nostrils (except for one dromedary) and for EDTA blood and serum. All vaccinated dromedaries seroconverted from day 16 after vaccination until 4 months shown by Rose-Bengal test and slide-agglutination test. No serological reactions were found after 5 months. The complement fixation test remained negative throughout the experiment. Information about the vaccination against brucellosis in camels, the within host disperse of the vaccine strain and the serological response are scarce. The experiment provided basic data about the feasibility of Brucevac conjunctival vaccine in camels. However, to prove if the immunised dromedaries acquired a lifelong immunity against brucellosis, pregnant vaccinated dromedaries need to be challenged with a field *B. melitensis* strain. We also recommend changing the conjunctival vaccination route to subcutaneous or intramuscular to prevent accidental infection due to *B. melitensis* vaccine strain excreted by lacrimation.

Key words: *Brucella melitensis* Rev 1, dromedary brucellosis, eye drop vaccination

Brucellosis remains wide spread in domestic and wild animal populations and presents a great economic burden for tropical animal husbandry (Seifert, 1992). It is also one of the most important zoonosis in developing countries with more than 500,000 new cases annually worldwide (WHO/FAO, 1986). Infection prevalence in animal reservoirs determines the incidence of human cases (Von Hieber, 2010). Old World camels are frequently infected with brucellosis especially, with *Brucella (B.) melitensis*, particularly when they are in contact with infected small ruminants (Wernery, 2014). The disease is rare in new world camels but outbreaks with classical signs of brucellosis have been described (Fowler, 2010).

Serious efforts have been made to prevent the infection through the use of vaccines. In old world camels, both inactivated and attenuated *Brucella* vaccines have been used successfully with both *B. abortus* strain S19 (Agab *et al*, 1995) and with *B. melitensis* (Radwan *et al*, 1995). However, so far no challenge infections have been performed in pregnant vaccinated dromedaries (*Camelus dromedarius*).

We herewith, describe laboratory investigations after single eye drop immunisation of 6 dromedaries with a live attenuated *B. melitensis* Rev 1 vaccine.

Materials and Methods

Eight dromedaries were selected for this study of which 6 were immunised and two were kept as control/contact animals. The camels were kept in 2 outdoor pens of the Central Veterinary Research Laboratory (CVRL, Dubai) in shaded areas, with 4 camels in each pen. The dromedaries were of different gender and age (Table 1) and received daily alfalfa hay *ad libitum* and 2 kg of concentrate per animal.

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All dromedaries had free access to water. None of the female dromedaries was pregnant. The welfare of all experimental animals and treatment of them conducted by CVRL were reviewed and approved by the Animal Ethic Committee of CVRL and Ministry of Climate Change and Environment of the United Arab Emirates (permit number: 550353).

Table 1. Dromedaries vaccinated with *B. melitensis* Rev 1.

Camel ID	Gender	Age in Years	Trial Category
CA-1	Female	16	Control
CA-2	Female	12	Vaccinated
CA-3	Female	18	Vaccinated
CA-4	Female	14	Vaccinated
CA-5	Male	9	Control
CA-6	Male	10	Vaccinated
CA-7	Male	11	Vaccinated
CA-8	Female	10	Vaccinated

The vaccine used in the study was 'Brucevac', a freeze dried conjunctival live attenuated *Brucella melitensis* strain Rev 1 developed by JOVAC (Jordan Bioindustries Limited, Jordan). It has a titre of 3.1 × 10^9 colony forming unit (CFU) of live attenuated *Brucella melitensis* strain Rev 1 per drop. The recommended dosage is one drop per animal. The vaccine was stored refrigerated and reconstituted as per manufacturer's instructions prior to use.

Each of the 6 selected experimental dromedaries (Table 1) received a single dose of the eye drop vaccine into the right conjunctival sac. One drop consisted of approximately 40 µl. Prior to immunisation, swabs were collected from all the 8 dromedaries and thereafter on 2nd, 4th, 10th, 16th and 24th day post immunisation. Right eye, left eye, right nostril and left nostril of all 8 dromedaries were swabbed using separate sterile cotton tipped swabs. After collection, swabs were immediately placed into 100 µl of tryptic soy broth Tryptic Soy Broth (TSB), (Merck 1.05459.05000) with *Brucella* selective supplements (Oxoid SR0083A). Blood was collected in EDTA tubes before and after immunisation (on 2nd, 4th and 10th day). Serum samples were collected on 2nd, 4th, 10th, 16th, 24th day post immunisation and thereafter, monthly for 5 months from all 8 camels. EDTA blood samples were directly frozen at -80°C. The swabs and serum samples were processed on the same day of collection and were stored at -80°C.

All swabs were streaked onto 2 selective and a non-selective media: Farrell's media (Oxoid CM0169) Brain Heart Infusion agar (Oxoid CM1135 with 1% added agar) with *Brucella* selective supplements (Oxoid SR0083A) and Tryptic Soy agar (Merck 1.05459.05000 with 1.5% added agar). All plates were incubated for 12 days at 37°C in 5% CO_2 atmosphere. After 12 days the plates were examined for the presence of *Brucella* bacteria and suspicious colonies counted/graded and recorded. The suspicious colonies were preliminarily identified as *Brucella* sp. by their growth characteristics on selective agars, Gram reaction, catalase and oxidase tests. The sera were tested for *Brucella* antibodies using the OIE described test procedures; complement fixation test (CFT), Rose Bengal test (RBT) and serum agglutination test (SAT) (World Organisation for Animal Health, 2016). RBT antigen, CFT antigen and SAT antigen were purchased from Animal Health and Plant Agency, Weybridge, UK. A serum containing 30 or more IU per ml was considered to be positive in SAT (World Organisation for Animal Health, 2016). DNA was extracted from the samples with the QIAamp DNA Mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. The samples were examined for the presence of *Brucella* species with the qPCR assay targeting the bcsp31 gene using the primers bcsp31F (5'-GCT CGG TTG CCA ATA TCA ATG C-3') and bcsp31R (5'-GGG TAA AGC GTC GCC AGA AG-3'), and the probe bcsp31P (5'-FAM-AAA TCT TCC ACC TTG CCC TTG CCA TCA-BHQ1-3') (Probert *et al*, 2004). The reference strain *B. suis* biovar 2 Thomsen (ATCC 23445) was used as positive control during the examinations. All samples were run in duplicate.

Results

Detailed culture, PCR and serological results are shown in tables 2 and 3 after eye drop immunisation with a commercial *B. melitensis* Rev 1 live vaccine in dromedaries. All swab and blood samples were negative for *Brucella* by culture, PCR assays and serological tests before vaccination. Also the 2 negative control animals in pens remained negative throughout the experiment.

B. melitensis bacteria grew on all 3 culture media from the right eye from all vaccinated dromedaries from 2nd day onwards until 10th (5 animals) to 16th (animal ID: CA3) day post immunisation (p.i.). Swabs of the left eye and both nostrils remained culture negative throughout the experiment.

Swabs of the right eye examined by PCR tests became positive from 2nd day onwards until 10th (5 animals) to 16th (animal ID: CA3) day p.i. Only one swab sample of the right nostril swabs showed positivity by PCR on day 2 p.i., dromedary CA3.

Table 2. Culture and PCR results after eye drop immunisation with *B. melitensis* Rev 1 of dromedary camels.

Days Post Immunisation	Camel ID	Bacteriology - Culture				PCR –CT Values (Duplicate run)					
		Eye Swab		Nostril Swab		Eye Swab		Nostril Swab		EDTA Blood	Serum
		Right	Left	Right	Left	Right	Left	Right	Left		
1	2	3	4	5	6	7	8	9	10	11	12
Before Immunisation	CA-1 (Control)	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
	CA-5 (Control)	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
	CA-2	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
	CA-3	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
	CA-4	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
	CA-6	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
	CA-7	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
	CA-8	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
2 days Post Immunisation	CA-1 (Control)	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
	CA-5 (Control)	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
	CA-2	POS(+)	NEG	NEG	NEG	37.44 | 38.29	NEG	NEG	NEG	NEG	NEG
	CA-3	POS(+)	NEG	NEG	NEG	30.92 | 30.59	NEG	38.42 | 37.78	NEG	NEG	NEG
	CA-4	POS(+)	NEG	NEG	NEG	29.61 | 29.44	NEG	NEG	NEG	NEG	NEG
	CA-6	NEG	NEG	NEG	NEG	34.84 | 34.21	NEG	NEG	NEG	NEG	NEG
	CA-7	POS(+)	NEG	NEG	NEG	33.41 | 33.51	NEG	NEG	NEG	NEG	NEG
	CA-8	POS(+)	NEG	NEG	NEG	30.72 | 31.28	NEG	NEG	NEG	NEG	NEG
4 days Post Immunisation	CA-1 (Control)	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
	CA-5 (Control)	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
	CA-2	POS(++)	NEG	NEG	NEG	38.15 | NEG	NEG	NEG	NEG	NEG	NEG
	CA-3	POS(++)	NEG	NEG	NEG	31.17 | 28.86	NEG	NEG	NEG	NEG	NEG
	CA-4	POS(+++)	NEG	NEG	NEG	30.94 | 31.75	NEG	NEG	NEG	NEG	NEG
	CA-6	POS(++)	NEG	NEG	NEG	31.9 | 32.17	NEG	NEG	NEG	NEG	NEG
	CA-7	POS(++)	NEG	NEG	NEG	35.06 | 33.82	NEG	NEG	NEG	NEG	NEG
	CA-8	POS(+++)	NEG	NEG	NEG	33.66 | 33.89	NEG	NEG	NEG	NEG	NEG
10 days Post Immunisation	CA-1 (Control)	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
	CA-5 (Control)	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
	CA-2	POS(+)	NEG	NEG	NEG	37.93 | NEG	NEG	NEG	NEG	NEG	NEG
	CA-3	POS(+)	NEG	NEG	NEG	37.61 | 36.89	NEG	NEG	NEG	NEG	NEG
	CA-4	POS(+)	NEG	NEG	NEG	NEG | NEG	NEG	NEG	NEG	NEG	NEG
	CA-6	POS(+)	NEG	NEG	NEG	NEG | NEG	NEG	NEG	NEG	NEG	NEG
	CA-7	POS(+)	NEG	NEG	NEG	NEG | NEG	NEG	NEG	NEG	NEG	NEG
	CA-8	POS(+)	NEG	NEG	NEG	NEG | NEG	36.9	NEG	NEG	NEG	NEG
16 days Post Immunisation	CA-1 (Control)	NEG	NEG	NEG	NEG	NEG | NEG	NEG	NEG	NEG	NEG	NEG
	CA-5 (Control)	NEG	NEG	NEG	NEG	NEG | NEG	NEG	NEG	NEG	NEG	NEG
	CA-2	NEG	NEG	NEG	NEG	NEG | NEG	NEG	NEG	NEG	NEG	NEG
	CA-3	POS(+)	NEG	NEG	NEG	34.71 | 35.23	NEG	NEG	NEG	NEG	NEG
	CA-4	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
	CA-6	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
	CA-7	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
	CA-8	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG

1	2	3	4	5	6	7	8	9	10	11	12
24 days Post Immunisation	CA-1 (Control)	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
	CA-5 (Control)	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
	CA-2	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
	CA-3	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
	CA-4	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
	CA-6	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
	CA-7	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
	CA-8	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG

Culture Key: + (1-50 colonies per plate), ++ (51-150 colonies per plate), +++ (> 150 colonies per plate)

Table 3. Serology results after eye drop immunisation with *B. melitensis* Rev 1 of dromedary camels.

Days Post Immunisation	Camel ID	Serology			Camel ID	Serology		
		RBT	CFT	SAT		RBT	CFT	SAT
1	2	3	4	5	6	7	8	9
Before Immunisation	CA-1 (Control)	NEG	NEG	NEG	CA5 (Control)	NEG	NEG	NEG
	CA-2	NEG	AC*	NEG	CA-6	NEG	NEG	NEG
	CA-3	NEG	NEG	NEG	CA-7	NEG	NEG	NEG
	CA-4	NEG	NEG	NEG	CA-8	NEG	NEG	NEG
2 days Post Immunisation	CA-1 (Control)	NEG	NEG	NEG	CA-5 (Control)	NEG	NEG	NEG
	CA-2	NEG	AC*	NEG	CA-6	NEG	NEG	NEG
	CA-3	NEG	NEG	NEG	CA-7	NEG	NEG	NEG
	CA-4	NEG	NEG	NEG	CA-8	NEG	NEG	NEG
4 days Post Immunisation	CA-1 (Control)	NEG	NEG	NEG	CA-5 (Control)	NEG	NEG	NEG
	CA-2	NEG	AC*	NEG	CA-6	NEG	NEG	NEG
	CA-3	NEG	NEG	NEG	CA-7	NEG	NEG	NEG
	CA-4	NEG	NEG	NEG	CA-8	NEG	NEG	NEG
10 days Post Immunisation	CA-1 (Control)	NEG	NEG	NEG	CA-5 (Control)	NEG	NEG	NEG
	CA-2	NEG	AC*	NEG	CA-6	NEG	NEG	NEG
	CA-3	NEG	NEG	NEG	CA-7	NEG	NEG	NEG
	CA-4	NEG	NEG	NEG	CA-8	NEG	NEG	Doubtful 26.5 IU/ml
16 days Post Immunisation	CA-1 (Control)	NEG	NEG	NEG	CA-5 (Control)	NEG	NEG	NEG
	CA-2	POS (1+)	AC*	POS 80 IU/ml	CA-6	POS (2+)	NEG	POS 268 IU/ml
	CA-3	NEG	NEG	POS 80 IU/ml	CA-7	POS (4+)	NEG	POS 424 U/ml
	CA-4	POS (2+)	NEG	POS 424 IU/ml	CA-8	POS (4+)	NEG	POS 424 IU/ml
24 days Post Immunisation	CA-1 (Control)	NEG	NEG	NEG	CA-5 (Control)	NEG	NEG	NEG
	CA-2	POS (2+)	AC*	POS 134 IU/ml	CA-6	POS (3+)	NEG	POS 424 IU/ml
	CA-3	POS (4+)	NEG	POS 268 IU/ml	CA-7	POS (4+)	NEG	POS 424 IU/ml
	CA-4	POS (4+)	NEG	POS 424 IU/ml	CA-8	POS (4+)	NEG	POS 424 IU/ml
43 days Post Immunisation	CA-1 (Control)	NEG	NEG	NEG	CA-5 (Control)	NEG	NEG	NEG
	CA-2	POS (2+)	AC*	POS 67 IU/ml	CA-6	POS (4+)	NEG	POS 268 IU/ml
	CA-3	POS (4+)	NEG	POS 160 IU/ml	CA-7	POS (4+)	NEG	POS 268 IU/ml
	CA-4	POS (4+)	NEG	POS 320 IU/ml	CA-8	POS (4+)	NEG	POS 268 IU/ml

1	2	3	4	5	6	7	8	9
73 days Post Immuni-sation	CA-1 (Control)	NEG	NEG	NEG	CA-5 (Control)	NEG	NEG	NEG
	CA-2	NEG	NEG	Doubtful 23.25 IU/ml	CA-6	POS(2+)	NEG	POS 134 IU/ml
	CA-3	POS(2+)	NEG	POS 80 IU/ml	CA-7	POS(3+)	NEG	POS 134 IU/ml
	CA-4	POS(4+)	NEG	POS 160 IU/ml	CA--8	POS(3+)	NEG	POS 186 IU/ml
3 months Post Immuni-sation	CA-1 (Control)	NEG	NEG	NEG	CA-5 (Control)	NEG	NEG	NEG
	CA-2	NEG	NEG	NEG	CA-6	NEG	NEG	Doubtful 26.5 IU/ml
	CA-3	NEG	NEG	POS 46.5 IU/ml	CA-7	NEG	NEG	Doubtful 26.5 IU/ml
	CA-4	POS(1+)	NEG	POS 186 IU/ml	CA-8	POS(1+)	NEG	POS 134 IU/ml
4 months Post Immuni-sation	CA-1 (Control)	NEG	NEG	NEG	CA-5 (Control)	NEG	NEG	NEG
	CA-2	NEG	NEG	NEG	CA-6	NEG	NEG	Doubtful 26.5 IU/ml
	CA-3	NEG	NEG	Doubtful 26.5 IU/ml	CA-7	NEG	NEG	Doubtful 26.5 IU/ml
	CA-4	POS(1+)	NEG	POS 80 IU/ml	CA8	POS(1+)	NEG	POS 134 IU/ml
5 months Post Immuni-sation	CA-1 (Control)	NEG	NEG	NEG	CA-5 (Control)	NEG	NEG	NEG
	CA-2	NEG	NEG	NEG	CA-6	NEG	NEG	NEG
	CA-3	NEG	NEG	NEG	CA-7	NEG	NEG	NEG
	CA--4	NEG	NEG	NEG	CA-8	NEG	NEG	NEG

RBT agglutination Key: + Dubious, ++ Positive, +++ Strong positive, ++++ Very strong positive

*AC :Anticomplementary reaction

Swabs of the left eye and nostril, as well as EDTA blood and serum samples remained negative by PCR throughout the experiment.

RBT and SAT showed first positivity on day 16 p.i. and remained positive for 4 months with different strength as shown in table 3. Only one camel (animal ID: CA8) was dubious in SAT already on day 10 p.i. CFT remained negative throughout the entire experimental period of 5 months.

Discussion

The information is limited about vaccination against brucellosis in camels, the optimal vaccination age, the dissemination of the vaccine strain and the serological response. Dromedaries were vaccinated with *B. abortus* strain S19 (Agab *et al*, 1995) and with *B. melitensis* in previous studies (Radwan *et al*, 1995). Agab *et al* (1995) vaccinated 5 dromedaries subcutaneously with a reduced dose (5×10^8 CFU/2 ml) of *B. abortus* strain S19. All 5 camels sero converted (RBT, SAT, cELISA) after 1 week and their antibody level declined after 7 weeks and the animals were tested negative 14 weeks later. Radwan *et al* (1995) vaccinated 3 month old dromedaries with a full dose (1.2×10^9 CFU/ml) of *B. melitensis* Rev 1 vaccine subcutaneously and adults above 10 years with a reduced dose (1.2×10^6 CFU/ml) subcutaneously. Both groups developed *Brucella* specific antibodies with titres between 1:25 and 1:200 using the standard USDA BPAT (made from *B. abortus* strain 1119-3), 2-4 weeks after vaccination. The antibodies receded after 8 months in young stock and after 3 months in adult camels. Similar results were obtained in our study, showing the decline of antibody level in adult camels 5 months after immunisation by RBT and SAT (similar to the USDA BPAT). The reason for the negative CFT throughout the experiment was not clarified, but it is hypothesised that the attenuation of the Rev 1 bacteria may have caused this phenomenon.

The attenuated vaccine, *B. melitensis* Rev 1 is used worldwide and it gives full immunity in sheep and goats by the conjunctival route with a dose of 1.0 to 2.0×10^9 CFU/animal. A slightly higher dose of 3.1×10^9 CFU/dromedary was used in our vaccination trial and the results showed that the *B. melitensis* vaccine strain was viable in the conjunctival sac of vaccinated animals for 10 to 16 days. Our conjunctival dromedary vaccination experiment did not include the testing to prove lifelong immunity in camelids as no challenge infections have been performed. Camels have a physiological constant lacrimation to clean their eyes from sand and dust, and by shedding the vaccine strain through their tears they may infect humans and other animals. Human infection with Rev 1 after consuming milk from vaccinated adult pregnant

animals was reported before (Bradenstein *et al*, 2002). Although, none of the control dromedaries became brucellosis positive in our study, we recommend not to vaccinate camelids through the conjunctival route but subcutaneously or intramuscularly due to the long eye excretion period. If the subcutaneous or intra muscular routes are used, great care should be taken as in some cases, human brucellosis was inflicted from accidental self-inoculation with live vaccine (Saleem *et al*, 2010).

An eradication campaign in camelids may be based on vaccination and 'test and slaughter' policy for dairy herds and 'test and no breeding' for racing herds (Wernery, 2014), because vaccinations alone would not suffice for success. The main approach in a long term control strategy of camelid brucellosis is to vaccinate only 1 to 2 year-old female replacement camels. An immunised herd could be established by this strategy without inducing abortion and excreting the vaccine strain through milk.

Conclusion

Brucella melitensis Rev 1 vaccine strain was isolated for 10 to 16 days from the right eye of 6 dromedary camels after conjunctival immunisation and may therefore be a risk for other animals as well as humans. Therefore, a subcutaneous or intramuscular immunisation is recommended.

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