Molecular and serological detection of *A. centrale*- and *A. marginale*-infected cattle grazing within an endemic area

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Abstract

A reverse line blot hybridization (RLB) one-stage nested PCR (nPCR) for *Anaplasma centrale* and a nested PCR for *Anaplasma marginale* were used to detect infected cattle grazing within an endemic region in Israel. A novel set of PCR primers and oligonucleotide probes based on a 16S ribosomal RNA gene was designed for RLB detection of both *Anaplasma* species, and the performance of the molecular assays compared. The immunofluorescent antibody test (IFA) was used to detect antibodies to both *Anaplasma* species, whereas, a highly sensitive and specific competitive enzyme-linked immunosorbent assay (cELISA) was used to detect antibodies in *A. centrale*-vaccinated cattle. The RLB and the nested PCR procedures showed bacteremia with sensitivity of 50 infected erythrocytes per milliliter. Up to 93% of the *A. centrale* vaccinates carried specific antibodies that were detected by cELISA, and up to 71% of the vaccinated cattle were found to be naturally infected with *A. marginale* according to the PCR and the RLB assays. Nevertheless, no severe outbreaks of *A. marginale* infection occurred among vaccinated herds in this endemic region. It appears that both, molecular tools and serology are useful for evaluation of the vaccine efficacy. In the light of wide natural field infection with *A. marginale*, strong recommendations to continue the *A. centrale* vaccination program regime will continue until a new generation of non-blood-based vaccine will be developed.

Article Outline

1. Introduction

Anaplasma, a member of the Anaplasmataceae family (Dumler et al., 2001), is an intraerythrocytic tick-borne pathogen. Two species of *Anaplasma* that infect cattle are *Anaplasma marginale* (Theiler, 1910) and *Anaplasma centrale* (Theiler, 1911). Acute anaplasmosis is characterized by severe anemia, abortion, weight loss, decreased milk production and death (Palmer, 1989 and Kocan et al., 2000). *A. centrale*, a closely related species or subspecies (Dumler et al., 2001 and Shkap et al., 2002b), causes only a mild form of anaplasmosis and is used for routine vaccination of cattle in several regions of the world, including Israel (Pipano et al., 1986). It is well documented that immunization with *A. centrale* does not prevent infection with *A. marginale*, but the severity of the disease is reduced and death is prevented (Theiler, 1911). Recently, the reverse line blot hybridization (RLB) technique was developed by Gubbels et al. (1999) for the simultaneous detection and differentiation of tick-borne pathogens and their species.

The aim of the present study was to develop and apply RLB to the simultaneous detection of *A. centrale* and *A. marginale* in cattle grazing within an endemic region in Israel. In addition, nested PCRs of higher sensitivity than previously described were developed for identification of both *Anaplasma* species, and performance of the molecular assays compared. The assays were performed along with the immunofluorescent fluorescent antibody (IFA) and the competitive enzyme-linked immunosorbent assay (cELISA) (Molloy et al., 2001). The results obtained confirm the utility of the molecular and the serological assays as valuable epidemiological tools. The results obtained indicate that, despite significant infection with *A. marginale*, the freedom from anaplasmosis outbreaks in Israel might be associated with the efficient program of vaccination with *A. centrale*.

2. Materials and methods

2.1. Isolates of *Anaplasma*

An *A. centrale* vaccine strain was obtained from South Africa in 1952 and has been used since then as the standard vaccine in Israel. The Israeli tailed and non-tailed *A. marginale* strains (Shkap et al., 2002a) were originally isolated from infected cattle grazing in an endemic area (Klopfer et al., 1968). The *A. marginale* Virginia isolate was obtained from Oklahoma State University, USA (De la Fuente et al., 2005). *A. marginale* and *A.*

centrale were maintained by passages in splenectomized cattle and kept as frozen stabilates, cryopreserved in dimethyl sulfoxide (DMSO) at −70 °C (Love, 1972).

2.2. Blood samples collected from experimental (A) and field (B) infection

(A) Two splenectomized calves (An1 and An2) shown to be free from *Anaplasma* infection demonstrated by nested PCR and IFA were used for the study. Calves An1 and An2 were inoculated intravenously with 10^8 infected erythrocytes of *A. centrale* and *A. marginale* (Virginia isolate), respectively. Blood samples were collected during acute infection, washed three times with phosphate-buffered saline (PBS) at pH 7.4 by centrifugation at 1200 × *g* for 15 min each time. The buffy coat was removed, and the erythrocytes in the final pellet were resuspended in PBS and stored pending use in PCR assays. One month after the infection calf An1 (infected with *A. centrale*) was inoculated with 10^5 infected erythrocytes from calf An2 (infected with *A. marginale*); and calf An2 (initially infected with *A. marginale*) was inoculated with 10^5 infected erythrocytes from calf An1 (initially infected with *A. centrale*). Blood samples were collected weekly for three months and stored at −70 °C until used.

(B) Blood samples ($n = 90$) were collected from a herd grazing on the Golan Heights in Israel, where *A. marginale* infection is endemic. The herd had been vaccinated with *A. centrale* three years previously, at the weaning age of 6–8 months. For PCR, blood was collected into vacutainer tubes containing sodium citrate or EDTA, and the samples were processed as described above and kept frozen at −70 °C until used.

All the experiments were approved by the Kimron Veterinary Institutional Animal Welfare Committee.

2.3. DNA extraction

DNA was extracted from thawed blood by means of the QIAamp DNA Blood Mini Kit (QIAGEN, USA). The purified DNA samples were examined for concentration with the NanoDrop spectrophotometer (USA) resuspended to a DNA concentration of 100 μg ml^{-1}, and stored at −20 °C. Blood samples obtained from calves experimentally infected with *Babesia bovis*, *Babesia bigemina* and *Theileria annulata*, and from a non-infected calf were processed identically and served as negative controls.

2.4. One-stage nested PCR for *A. centrale*

A one-stage nested PCR for *A. centrale* was based on the amplification of the *A. centrale mpb*-58 sequence, described by Shkap et al. (2002a). By using a second set of primers based on the *A. centrale msp*2 operon (GenBank accession number AY132307), another one-stage nested PCR was developed. The external primers were AC1826 (forward primer, nucleotide position 1757–1781) 5′-TTGTGGCTCTAGTCCCCCGGGGAG A-3′ and AC 2367 (reverse primer, complement of 2298–2323) 5′-AGACAAAGAACCCGGCGTAGCAGCTC-3′). The internal primers were CIS1925 (forward primer, nucleotide position 1910–1929) 5′-TTCTTGAGCAGGGGGATACC-3′

and CIS2157 (reverse primer, complement of 2142–2161) 5′-AGACCCGGCGGA AATACCAT-3′. The two rounds of amplification were separated by limiting the concentration of the external primers. PCR was performed in a final reaction volume of 25 μl containing 5–10 ng genomic DNA as a template, 5 ng of each external primer, 25 ng of each internal primer, 1× PCR Takara buffer containing, 2 mM $MgCl_2$, 0.2 mM of each deoxynucleotide triphosphate, and 1.25 U of Takara Ex-Taq. The cycling protocol comprised: preheating at 95° C for 3 min; 14 cycles of 98° C for 10 s, 65° C for 30 s and 72° C for 30 s; and 26 cycles of 98° C for 30 s, 60° C for 30 s and 72° C for 30 s; with a final extension at 72° C for 5 min. Reaction products were analyzed by agarose gel electrophoresis.

2.5. Cloning and sequencing of the *msp*1b of the Israeli A. marginale tailed strain

Primers used for cloning of the Israeli *A. marginale msp*1b tailed strain were based on the published sequences of *msp*1b (GenBank accession number M59845). The forward primer was (position 404–425) 5′-ATGACAGAAGACGACAAGCAAC-3’ and the reverse primer was 5′-AGTAACAATTGCTTGGTCGT-3′ (complement of 2359–2340). PCR was performed in a final reaction volume of 50 μl: the PCR amplification mix contained 5–10 ng of purified genomic DNA as template, 100 ng of each primer, 1× PCR Takara buffer containing, 2 mM $MgCl_2$, 0.2 mM of each deoxynucleotide triphosphate, and 1.25 U of Takara Ex-Taq. The cycling protocol, following preheating at 95° C for 3 min was: 35 cycles of denaturation at 98° C for 10 s, annealing at 56° C for 45 s, and extension at 72° C for 1.30 min; with a final extension at 72° C for 15 min. PCR products were purified with the QIAQuick gel extraction kit (QIAGEN, USA) and ligated into pCR-TOPO-XL by using the cloning vector (Invitrogen, USA). Plasmid DNA was extracted from the recombinant clones and sequenced in both directions.

2.6. Nested PCR for *A. marginale*

Nested PCR (nPCR) for *A. marginale* was developed on the basis of the gene sequence of the Israeli tailed strain of *A. marginale msp*1b (GenBank accession no. AY841153). The external primers were AM456 (forward primer, nucleotide position 53–76) 5′-CCA TCTCGGCCGTATTCCAGCGCA-3′ and AM1164 (reverse primer, complement of 784–761) 5′-CTGCCTTCGCGTCGATTGCTGTGC-3′. The internal primers were AM100 (forward primer, nucleotide position 337–356)5′-CAGAGCATTGACGCACTA CC-3′ or AM219 (forward primer, nucleotide position 216–235) 5′-GGCAGATGCTAA ATACGAGA-3′ and AM101 (reverse primer, complement of 582–563) 5′-TTCCAG ACCTTCCCTAACTA-3′. The PCR was performed in a final reaction volume of 25 μl, the PCR amplification mix contained 5–10 ng of purified genomic DNA as template, 1× PCR Takara buffer containing, 2 mM $MgCl_2$, 100 ng of each primer, 0.2 mM of each deoxynucleotide triphosphate and 1.25 U of Takara Ex-Taq. The cycling protocol comprised an initial 3 min of denaturizing at 95° C, followed by 30 cycles of 10 s denaturation at 98° C and 30 s annealing at 60° C, followed by 30 s elongation at 72° C with a final extension at 72° C. For nested amplification 1 μl of the primary PCR product was used as a template in a total volume of 25 μl. Nested cycling conditions were as

described for the primary amplification, except that the annealing was at 54° C. Reaction products were analyzed by agarose gel electrophoresis.

2.7. Reverse line blot hybridization assay

The PCR reaction for RLB involved one set of primers designed to amplify the *A. marginale* and *A. centrale* 16S ribosomal RNA genes (GenBank accessions AF414875 for *A. marginale*, and AF309869 for *A. centrale*). The forward primer was RLB-F790 (biotin-5′-GGCTTTTGCCTCTGTGTTGT-3′) corresponding to nucleotides 792–811 and 793–812 for *A. marginale* and *A. centrale*, respectively. The reverse primer used was RLB-R1134 (biotin-5′-CTTGACATCATCCCCACCTT-3′), corresponding to nucleotides 1154–1135 and 1155–1136 for *A. marginale* and *A. centrale*, respectively. Reaction condition in 25 μl volume were as follows: each reaction mixture contained 50 pmol of primers RLB-F790 and RLB-R1134, 0.25 μg of the TaqStart antibody (Clontech, USA), 0.1 U of uracil-DNA glycosylase (UDG) (Gibco BRL, USA), deoxynucleoside triphosphates (200 μM dATP, dGTP, dCTP, 100 μM dUTP and 100 μM dTTP), 1× SuperTaq buffer (10 mM Tris–HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% stabilizer, 0.1% Triton X-100), 1.25 U of SuperTaq DNA polymerase (HT Biotechnology, Ltd., Cambridge, UK) and 2 μl of DNA as template. The mixture was incubated for 3 min at 37° C to promote UDG activity, followed by 10 min incubation at 94° C to inactivate the UDG. A touchdown PCR program was performed: two cycles of 20 s at 94° C, 30 s at 65° C and 30 s at 72° C, and afterwards two cycles with conditions identical to the previous cycles, but with an annealing temperature of 63° C. In each of the subsequent pairs of cycles the annealing temperature was lowered by 2° C until it reached 57° C. A further 40 cycles each comprised 20 s at 94° C, 30 s at 57° C and 30 s at 72° C, and there was a final extension at 72° C for 7 min. The hybridization was performed as previously described by Gubbels et al. (1999). Briefly, the two oligonucleotide probes provided bellow (subtitle 3.2) contained an N-terminal *N*-(tri-fluroacetamidohexyl-cyanoethyl,*N*,*N*-diisopropyl phosphoramidite [TFA])-C_6 amino linker (PROLIOGO, USA). A Biodyne C blotting membrane (Pall Corporation, USA) was activated for 30 min with 10% 1⁻ethyl⁻3⁻ (3⁻dimethylamino-propyl) carbodiamide (EDAC) (Sigma, USA) at room temperature. The membrane was placed in an MN 45 miniblotter (Immunetics, Cambridge,). The oligonucleotide probes were diluted to concentrations of 400 or 500 pmol per 150 μl, in 500 mM $NaHCO_3$ (pH 8.4) and were subsequently covalently linked to the membrane. The reaction was visualized by enhanced chemiluminesence (ECL) detection liquid (Amersham Biosciences) and exposure to an ECL hyperfilm.

2.8. Sensitivity of the nested PCR and RLB assays

The sensitivity of the assays was determined by tenfold serial dilution of 5×10^9 *Anaplasma*-infected erythrocytes with blood that contained uninfected erythrocytes. All samples were adjusted to a final erythrocyte concentration of 5×10^9 cells ml^{-1}. The DNA extracted from each sample was amplified as described above.

2.9. Serology

The IFA test was used to detect antibodies to *A. marginale* and *A. centrale* as described by Goldman et al. (1972) and Shkap et al. (1990). Highly specific cELISA for identification of *A. centrale* antibodies in vaccinated cattle was performed as described by Molloy et al. (2001).

3. Results

3.1. Specificity and sensitivity of nested PCR for A. marginale and A. centrale

Amplification of the Israeli tailed *A. marginale msp*1b sequence resulted in a 2274-bp product that was cloned and sequenced (GenBank accession number AY841153). Based on these sequences PCR primers for nPCR were designed. Amplification of *A. marginale* by nPCR with the internal primers AM100 + AM101 resulted in detection of a 246-bp product (Fig. 1, lanes 1–4), or of a 367-bp product if the second internal primers were used AM219 + AM101 (not shown). The nPCR detection limit for *A. marginale* (for the two sets of primers) was 50 infected erythrocytes per milliliter (Fig. 1, lane 4). No PCR products were amplified when DNA from *B. bovis*, *B. bigemina* or *A. centrale* was used (Fig. 1, lanes 6–8, respectively). The specificity and detection limit of the one-stage nested PCR for *A. centrale*, based on the *mpb*-58 sequence were determined previously in another study (Shkap et al., 2002a); the detection limit was found to correspond to 500 infected erythrocytes per milliliter. To increase the sensitivity of *A. centrale* detection, a second one-stage nested PCR assay with primers based on the *A. centrale msp*2 operon was developed. Amplification of *A. centrale* resulted in a specific 252-bp fragment with a detection limit corresponding to 50 infected erythrocytes (not shown).

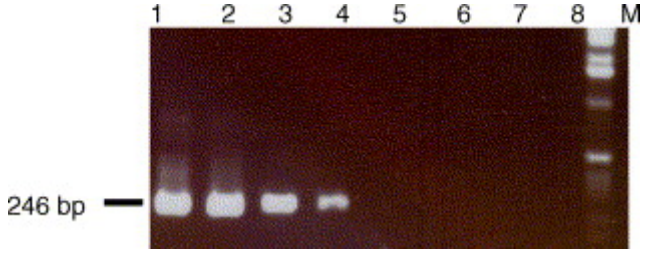

Fig. 1. Sensitivity and specificity of *A. marginale* detection by nested PCR. DNA was extracted from blood containing *Anaplasma*-infected erythrocytes and was amplified by PCR. Amplification that resulted from tenfold dilution of *A. marginale*-infected erythrocytes is shown in lanes 1–5. Lane 1, 5×10^4; lane 2, 5×10^3; lane 3, 5×10^2; lane 4, 5×10^1; lane 5, 0.5×10^1; lane 6, *B. bovis*; lane 7, *B. bigemina*; lane 8, 5×10^4 *A. centrale;* lane M, 1 kb ladder.

3.2. RLB-specific oligonucleotide probes

The nucleotide sequences and location of the two *Anaplasma* spp. oligonucleotide probes based on the 16S rRNA gene: *A. centrale* (position 955–974 bp) ACCTTACCACTTCTTGACATGGAGGCTAGATCTTCCTTAACGGGAAGGCGCA GTTCGGCT and *A. marginale* (position 953–975 bp) ACCTTACCACTTCTTGACATGGAGGCTAGATCCTTCTTAACAGAAGGGCGCA GTTCGGCT.

3.3. Specificity and sensitivity of RLB

As shown in Fig. 2A RLB-PCR products of *A. centrale* and of the Israeli non-tailed and tailed *A. marginale* bound only to the specific oligonucleotide probes (lanes 3–4 and 5–6, respectively). Additionally, blood samples that were taken from the experimentally double-infected calves, An1 and An2, two and three months after the initial infection reacted with both species of *Anaplasma* (lanes 7–10). When PCR products of *B. bovis*, *B. bigemina* and *T. annulata* were applied to an RLB membrane (Fig. 2A, lanes 1–2, Fig. 3, lanes 2–4) no reaction with the oligonucleotides was observed. The sensitivity of the RLB assay for the detection of *A. centrale* and *A. marginale* was measured by tenfold serial dilution. The RLB assay revealed a detection threshold of 50 infected erythrocytes, both for *A. centrale* (Fig. 2B) and *A. marginale* (not shown).

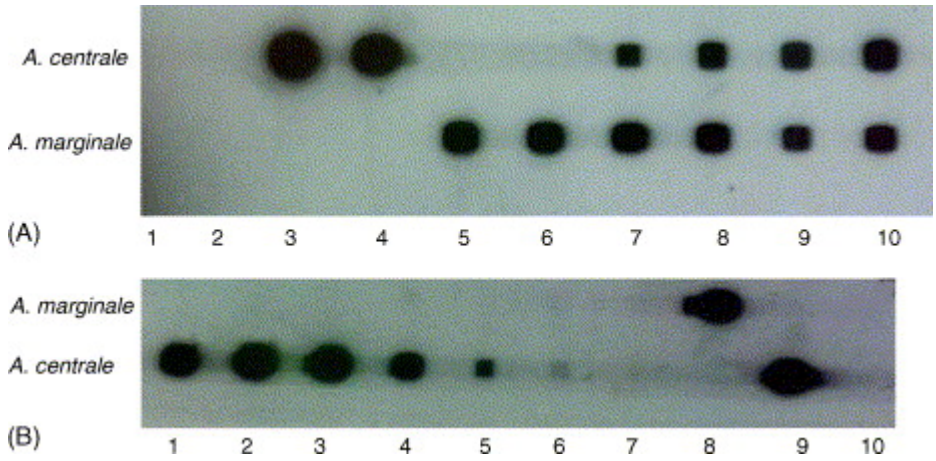

Fig. 2. (A) Specific detection of *A. centrale* and *A. marginale* by RLB. PCR products were applied in vertical lanes. Species-specific oligonucleotide probes were applied in horizontal rows. Lane 1, *B. bovis*; lane 2, *B. bigemina*; lanes 3 and 4, *A. centrale*; lane 5, *A marginale* Israeli non-tailed strain; lane 6, *A. marginale* Israeli tailed strain; lanes 7 and 8, calf An1 two and three months after primary infection with *A. marginale*; lanes 9 and 10, calf An2 two and three months after infection with *A. centrale*. (B) Sensitivity of *A. centrale* detection by RLB assay. Seven tenfold dilution of *A. centrale*-infected erythrocytes is shown in lanes 1–7. Lane 1, 5×10^6; lane 2, 5×10^5; lane 3, 5×10^4; lane 4, 5×10^3; lane 5, 5×10^2; lane 6, 5×10^1; lane 7, 0.5×10^1; lane 8, *A. marginale* Israeli tailed strain (10^9 infected erythrocytes); lane 9, *A. centrale* (10^9 infected erythrocytes); lane 10, DNA from *B. bovis*.

Fig. 3. Detection of *Anaplasma*-carrier cattle within a herd in an endemic region in Israel, by RLB. PCR products were applied in vertical lanes. Species-specific oligonucleotide probes were applied in horizontal rows. Lane 1, uninfected calf; lane 2, *T. annulata*; lane 3, *B. bovis*; lane 4, *B. bigemina*; lane 5, *A. centrale*; lane 6, *A. marginale*; lanes 7–23: blood samples collected from the field.

3.4. Detection of *Anaplasma* in vaccinated herd grazing within an endemic region

As shown in Fig. 3 the RLB-PCR products of blood samples from the field-grazing vaccinated herd were hybridized to the membrane and further analyzed by RLB. The results of RLB were compared to those obtained with the nested PCR and serology assays (Table 1). Out of 90 samples, 64 reacted simultaneously with *A. marginale* and *A. centrale* oligonucleotide probes by RLB; 70 and 76 samples were positive by RLB or nPCR, for *A. marginale* and *A. centrale*, respectively. The serology results indicated that out of 90 animals examined, 76 and 84 were positive by IFA and cELISA, respectively. There was a discrepancy in the detection of *A. centrale* by molecular methods and the cELISA. Eight animals negative by RLB and one-stage nPCR were found positive by the cELISA. There were 64 RLB positive cattle carrying *A. centrale* and *A. marginale*, while 76 appeared positive in the IFA, which might show seropositivity to either species, or simultaneous *Anaplasma* infection (Table 1).

Table 1.

Detection of *Anaplasma* in vaccinated cattle grazing within an endemic region by molecular and serological methods ($n = 90$)

***Anaplasma* organisms**	**Numbers (%) of positively reacted cattle shown by molecular and serological assays**				
	One-stage nPCR	**nPCR**	**RLB**	**IFA[a]**	**cELISA**
A. marginale		70 (78)	70 (78)		
A. centrale	76 (84)		76 (84)		84 (93)
A. marginale and *A. centrale*			64 (71)	76 (84)	

[a] The IFA does not distinguish between antibodies to *A. marginale* and *A. centrale* (Shkap et al., 1990).

4. Discussion

In the present study molecular and serological assays were applied for the detection of persistently infected cattle that had been vaccinated with *A. centrale* or that were carrying *A. marginale* as a result of natural infection. For the RLB assay, specific PCR primers and species-specific oligonucleotide probes based on 16S rRNA were designed in this study. The RLB and the two PCR assays (one-stage and nested PCR) showed bacteremia of 50 infected erythrocytes per milliliter of blood. To increase the sensitivity from 500 to 50 infected cells described previously (Shkap et al., 2002a), another set of primers was designed, based on sequences of *A. centrale msp*2 operon. Similarly, a new set of primers based on *A. marginale msp*1b was applied for *A. marginale* nested PCR. RLB tests for detection of *Anaplasma* and *Ehrlichia* were successfully applied by Bekker et al. (2002), but the sensitivity of those assays was not specified. The molecular assays used in the present study were found to be highly specific; no reactivity was obtained with DNA samples from other commonly co-endemic tick-transmitted pathogens. Although similar detection levels were achieved by the nPCR and RLB assays, the RLB offers the advantage of simultaneous detection of both *Anaplasma* species, which is valuable for the determination of the epidemiological status of a vaccinated herd grazing within an area where anaplasmosis is endemic. Examination of 90 field samples revealed 100% correlation between the RLB results and the nested PCR assays. The high percentage of *A. centrale* carrier cattle (84%) confirmed our previous finding that a single vaccination resulted in long-term persistence of infection in the majority of the vaccinates (Krigel et al., 1992 and Shkap et al., 2002a). Similar to our previous finding, large proportions of the cattle grazing within the endemic area were carrying *A. marginale* (78%) and were co-infected with both species (71%). These results indicate that continuous natural infection with *A. marginale* takes place, but the cattle are protected by vaccination with *A. centrale,* as no outbreaks of clinical anaplasmosis were reported from this specific herd. The IFA tests showed that most of the vaccinated herd possessed *Anaplasma* antibodies. The IFA test, which did not discriminate between *Anaplasma* species because of antigenic similarity (Palmer, 1989 and Visser et al., 1992) were widely used for serodiagnosis of anaplasmosis (Goff et al., 1985, Montenegro-James et al., 1985 and Shkap et al., 1990). A commercial cELISA based on the MSP 5 surface protein has been developed for detection of antibodies against *A. marginale* (Knowles et al., 1996 and Torioni de Echaide et al., 1998) but, similarly to the IFA, it was found not to be applicable for discrimination between *A. centrale*-specific antibodies (Molloy et al., 1999 and De la Fuente et al., 2005). The IFA based on 80 kDa *A. centrale* antigen differentiated between *A. marginale* and *A. centrale* in patent infections only, while the cELISA based on 116 kDa antigen was highly sensitive and specific in carrier infections (Molloy et al., 2001). The cELISA developed by Molloy et al. (2001), was successfully used in this study to detect the *A. centrale*-specific antibodies produced against the vaccine strain (Molloy et al., 2001), three years after vaccination the vast majority (94%) of cattle were serologically positive. The discrepancies in positive by serology (cELISA), but negative by molecular methods might be associated with the ability to clear *Anaplasma* infection by individual animals, while carrying serum antibodies (Torioni de

Echaide et al., 1998). It appears that both, molecular tools and serology are useful for identifying vaccinated cattle. In the light of wide natural field infection with *A. marginale*, our strong recommendation is to continue the *A. centrale* vaccination program until a new generation of non-blood-based vaccine is developed.

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