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Department of Veterinary Pathobiology, Center for Veterinary Health Sciences, Oklahoma State University, Stillwater, OK, USA

Genetic Diversity of *Anaplasma marginale* Strains from Cattle Farms in the Province of Palermo, Sicily

J. de la Fuente[1,2,5], A. Torina[3], V. Naranjo[2], S. Caracappa[3], J. Vicente[2], A. J. Mangold[4], D. Vicari[3], A. Alongi[3], S. Scimeca[3] and K. M. Kocan[1]

Addresses of authors: [1]Department of Veterinary Pathobiology, Center for Veterinary Health Sciences, Oklahoma State University, Stillwater, OK 74078, USA; [2]Instituto de Investigación en Recursos Cinegéticos IREC (CSIC-UCLM-JCCM), Ronda de Toledo s/n, 13005 Ciudad Real, Spain; [3]Instituto Zooprofilattico Sperimentale della Sicilia, Via G. Marinuzzi no. 3, 90129 Palermo, Italy; [4]Instituto Nacional de Tecnología Agropecuaria, Estación Experimental Agropecuaria Rafaela, CC 22, CP2300 Rafaela, Santa Fe, Argentina; [5]Corresponding author: Tel.: 405 744 0372; fax: 405 744 5275; E-mail: jose_delafuente@yahoo.com, djose@cvm.okstate.edu

With 1 figure and 1 table

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Summary

Bovine anaplasmosis, caused by the tick-borne rickettsia *Anaplasma marginale*, is endemic in Sicily and results in economic loss to the cattle industry. This study was designed to characterize strains of *A. marginale* at the molecular level from cattle in the Province of Palermo, Sicily. Seropositivity of cattle ≥1 year old for *A. marginale* in the study area ranged from 62% to 100%. The observed prevalence of *A. marginale* infections in cattle herds ranged from 25% to 100%. Two predominant *A. marginale msp4* genotypes were found. A positive correlation was found between the prevalence of infection and the presence of *Rhipicephalus (Boophilus) annulatus*. Phylogenetic analysis of *msp4* sequences of European strains of *A. marginale* did not provide phylogeographical information. These results suggest that development of farm husbandry systems and vaccines for genetically heterogeneous populations of *A. marginale* are needed for control of anaplasmosis in this region of Sicily.

Introduction

Bovine anaplasmosis is caused by *Anaplasma marginale*, the type species of the genus *Anaplasma* (Rickettsiales: Anaplasmataceae). Ticks are biological vectors of *A. marginale* but the pathogen is often transmitted mechanically to susceptible cattle by blood-contaminated mouthparts of biting flies or fomites (reviewed by Kocan et al., 2003). These obligate intracellular organisms replicate in membrane-bound parasitophorous vacuoles in bovine erythrocytes. Both cattle and ticks become persistently infected with *A. marginale* and thus serve as reservoirs of infection (reviewed by Kocan et al., 2003).

Many geographical strains of *A. marginale* have been identified which differ in biology, genetic characteristics and transmissibility by ticks (reviewed by de la Fuente et al., 2001, 2005a). The genetic diversity of *A. marginale* strains has been characterized using 16S rDNA (Dumler et al., 2001) and major surface proteins (MSPs) genes that are involved in interactions with vertebrate and invertebrate host cells. These genes may have evolved more rapidly than other chromosomal genes because of selective pressures exerted by the host-immune system (de la Fuente et al., 2005a).

The *A. marginale msp4* was posited to be a stable marker for the genetic characterization of strains because the protein does not appear to undergo antigenic variation in mammals or ticks (de la Fuente et al., 2005a). The *A. marginale msp4* gene demonstrated sufficient sequence variation to support its use for characterization of geographical strains of the pathogen (de la Fuente et al., 2005a). Phylogeographical resolution was obtained at the regional level (de la Fuente et al., 2001, 2002, 2004a, 2005a) but not when an analysis of *A. marginale* strains occurring worldwide was conducted (de la Fuente et al., 2005a).

The control of tick-borne pathogens in cattle reservoirs impacts the epidemiology of anaplasmosis, improves animal production and reduces the challenge to food safety management by reducing the use of antibiotics (Collins and Wall, 2004). The production and maintenance of healthy cattle stocks require good husbandry practices such as stock selection and veterinary attention, which vary with the scale of the enterprise, the breeds being farmed and the epidemiology of the pathogens in the particular geographical region (Dehaumont, 2004).

Livestock production in smallholder farming systems is an important component of the agricultural economy in Sicily, and the conditions range from extensive pastoralist to intensive feeder and dairy systems (Caracappa, 1999). *Anaplasma marginale* is endemic in Sicily and has been identified as an economically important pathogen for cattle production in the island (Caracappa, 1999). *Anaplasma marginale* infection has been reported in cattle and ticks in Sicily and in other regions of Italy (Cringoli et al., 2002; Tassi et al., 2002; de la Fuente et al., 2005a,b). However, development and implementation of anaplasmosis control measures is dependent upon understanding the epidemiology of *A. marginale* in this region, including the characterization of the genetic diversity of strains from cattle farms.

[1]This work was conducted at Instituto de Investigación en Recursos Cinegéticos IREC (CSIC-UCLM-JCCM), Ronda de Toledo s/n, 13005 Ciudad Real, Spain and Intituto Zooprofilattico Sperimentale della Sicilia, Via G. Marinuzzi no. 3, 90129 Palermo, Italy.

The study reported herein was designed to characterize *A. marginale* strains from cattle in farms in the Province of Palermo, Sicily. *Anaplasma marginale* genotypes were determined at the molecular level using 16S rDNA and *msp4* gene sequences.

Materials and Methods

Study site and sample collection

Seven cattle farms in the Province of Palermo, Sicily, were included in the study (Table 1). Farms were selected for the study in an *A. marginale* endemic area that covered the main characteristics of cattle husbandry and production in Sicily, including beef and/or dairy producers, with indigenous and/or imported cattle breeds, under intensive and/or extensive management, and infested with different tick species.

All cattle ≥1 year old were included in the study. The other cattle in the study area were excluded from the analysis because they were under 1 year of age or were sent to market before the age of 1.5 years. Blood was collected from 160 cattle into sterile tubes with and without anticoagulant (lithium heparin) and maintained at 4°C until arrival at the laboratory. Plasma and serum were then separated after centrifugation and stored at −20°C.

Ticks were collected monthly during the summer (May to August) from infested cattle and stored in 70% ethanol at room temperature. Ticks were identified using morphological keys for Italian Ixodidae (Manilla, 1998). Cattle herds identified as free of ticks at collection time had been treated with acaricides using deltamethrin or amitraz.

Serological test for detection of antibodies against *A. marginale*

The anaplasmosis cELISA was performed using the *Anaplasma* Antibody Test Kit, cELISA from VMRD, Inc. (Pullman, WA, USA) following the manufacturer's instructions. This assay specifically detects the presence of serum antibodies that targets the MSP5 protein of *Anaplasma* spp. (Knowles et al., 1996). Per cent inhibition values > 30% were considered positive using the anaplasmosis cELISA (de la Fuente et al., 2003a).

DNA extraction, PCR and sequence analysis

DNA was extracted from blood samples using the GenElute Mammalian Genomic DNA Miniprep Kit (Sigma, St Louis, MO, USA). The DNA was resuspended in sterile distilled water and stored at −20°C until used in polymerase chain reactions (PCRs). The *Anaplasma* 16S rDNA and the *A. marginale* species-specific *msp4* (sensitivity of 5 copies *msp4*/ng DNA) PCRs were used for pathogen identification. The *msp4* PCR was used for genotyping *A. marginale* strains as reported previously (de la Fuente et al., 2002, 2003b, 2004a, 2005a). A fragment of 468 nucleotides comprising 16S rDNA positions −1 to 467 of the *A. marginale* Florida strain reference sequence (GenBank accession number AF309867) was amplified by PCR using oligonucleotide primers 16SANA-F (5′-CAG AGT TTG ATC CTG GCT CAG AAC G-3′) and 16SANA-R (5′-GAG TTT GCC GGG ACT TCT TCT GTA-3′). The *msp4* and 16S rDNA gene sequences were amplified from 1 μl (0.1–10 ng) DNA by PCR using 10 pmol of each primer in a 50-μl volume (1.5 mM $MgSO_4$, 0.2 mM dNTP, 1X AMV/Tfl 5X reaction buffer, 5 U *Tfl* DNA polymerase) employing the access reverse transcriptase (RT)-PCR system (Promega, Madison, WI, USA). Reactions were performed in an automated DNA thermal cycler (Eppendorf Mastercycler® personal, Westbury, NY, USA) for 35 cycles. PCR products were electrophoresed on 1% agarose gels to check the size of amplified fragments by comparison to a DNA molecular weight marker (1 kb Plus DNA Ladder; Promega).

Amplified 16S rDNA and *msp4* fragments were resin purified (Wizard; Promega) and cloned into pGEM-T vector (Promega) or used directly for sequencing both strands by double-stranded dye-termination cycle sequencing (Core Sequencing Facility, Department of Biochemistry and Molecular Biology, Noble Research Center, Oklahoma State University). When cloned, at least two independent clones were sequenced. Multiple sequence alignment was performed with

Table 1. *Anaplsma marginale* infection, tick species parasitizing cattle and characteristics of cattle farms included in the study

Farm	Breed	*n*[a]	Production	Management[b]	Tick species[c]	Observed prevalence of *A. marginale* infection (*n*)[d]	*msp4* PCR-positive cattle	*msp4* Genotypes[e]			
								I	II	III	IV
Mascarella Antonino	Brown Swiss	31/58	Dairy	Intensive	None	55 ± 17 (17)	13	13	0	0	0
Impastato Giacomo	Pezzata Rossa	12/38	Dairy/beef	Mixed	*Rb, Hm, Hl, Ba*	100 ± 0 (12)	11	9	2	0	0
Tripi Gaetano	Indigenous	32/80	Beef	Extensive	*Rb, Rs, Rt, Hl, Hm*	69 ± 16 (22)	15	12	2	1	0
Barone Salvatore	Brown Swiss	22/50	Dairy	Intensive	*Hm, Hl, Rb*	50 ± 21 (11)	5	2	3	0	0
Nuova Sicilia	Brown Swiss and indigenous (*Bos taurus*)	13/45	Dairy	Extensive	None	31 ± 25 (4)	2	2	0	0	0
Martorana Rosaria	Pezzata Rossa	36/70	Dairy/beef	Intensive	*Rb, Rs, Rt, Hm, Hl*	25 ± 14 (9)	4	4	0	0	0
Galati Piero	Pezzata Rossa	14/50	Dairy/beef	Mixed	*Rb, Rs, Rt, Hl*	36 ± 25 (5)	5	4	0	0	1
Total	–	160/391	–	–	–	50 ± 8 (80)	55	46	7	1	1

[a]Number of animals sampled for PCR analysis (cattle ≥1 year old)/total number of animals in the farm.
[b]Farm management conditions range from extensive pastoralist to intensive feeder and dairy systems (Caracappa, 1999).
[c]Abbreviations for tick species: *Rb*, *Rhipicephalus bursa*; *Rs*, *Rhipicephalus sanguineus*; *Rt*, *Rhipicephalus turanicus*; *Hl*, *Hyalomma lusitanicum*; *Hm*, *Hyalomma m. marginatum*; *Ba*, *Rhipicephalus (Boophilus) annulatus*.
[d]The observed prevalence of *A. marginale* infection in cattle was determined by 16S rDNA PCR and expressed as percent infection ± SE at 95% CI; *n*, number of positive animals.
[e]*A. marginale msp4* genotypes I–IV corresponds to sequences with Genbank accession numbers DQ000618–DQ000621 respectively.

the program AlignX (Vector NTI Suite, version 5.5; InforMax, North Bethesda, MD, USA) with an engine based on the Clustal W algorithm (Thompson et al., 1994). Nucleotides were coded as unordered, discrete characters with five possible character-states: A, C, G, T or N and gaps were coded as missing data. Phylogenetic analysis of European *A. marginale msp4* sequences was conducted using a distance-based (Kimura two parameter) neighbour-joining method followed by branch-swapping as implemented in Mega 2. Stability or accuracy of inferred topology(ies) were assessed via bootstrap analysis of 1000 iterations. *Msp4* sequences from Mexican, Brazilian, Argentinean, North American and Israeli strains were used for comparison with European strains.

Statistical analysis

The observed prevalence of *Anaplasma* infections in cattle herds was analysed by PCR and sequence analysis of 16S rDNA amplicons and these data were correlated statistically with the tick species parasitizing cattle on each farm. The statistical analysis was performed by chi-squared test using the SPSS 11.0 statistical program (SPSS Inc., Chicago, IL, USA). The differences were considered statistically significant when $P \leq 0.05$. The CI and SE at 95% confidence level of the prevalence of *Anaplasma* infections in cattle were calculated based on Martin et al. (1987).

Sequence accession numbers

The GenBank accession numbers for *msp4* and 16S rDNA sequences of *A. marginale* strains are DQ000618–DQ000621 and DQ000613–DQ000617 respectively.

Results and Discussion

Anaplasma marginale is endemic in Sicily where it causes economic loss to the cattle industry (Caracappa, 1999). Seropositivity of cattle ≥1 year old for *A. marginale* in the study area ranged from 62% to 100% (mean ± SD, 80 ± 16). The observed prevalence of *A. marginale* infections in cattle herds ranged from 25% to 100% (mean ± SE at 95% CI, 50 ± 8) (Table 1). *Anaplasma* infections were identified in 80 and 55 of the 160 cattle analysed by 16S rDNA and *msp4* PCR respectively (Table 1). Sequence analysis of the *Anaplasma* amplicons confirmed that cattle were infected with *A. marginale*. Differences in the results of the 16S rDNA and *msp4* PCRs may reflect the higher copy number of 16S rDNA genes, which results in the higher sensitivity of the 16S rDNA PCR (de la Fuente et al., 2005b). The discrepancies between serology and PCR results could be explained by the absence of detectable levels of bacteraemia in some samples, a finding particularly common among chronically infected carrier animals (reviewed by Kocan et al., 2004).

Six tick species were collected from infested cattle in the study area (Table 1). The most widely distributed species were *Rhipicephalus bursa* and *Hyalomma lusitanicum* (5/7 farms), followed by *Hyalomma m. marginatum* (4/7 farms), *Rhipicephalus turanicus* and *Rhipicephalus sanguineus* (3/7 farms) and *Rhipicephalus (Boophilus) annulatus* (1/7 farms). Two farms were tick-free during the sampling period, most likely because of the application of acaricides. All tick genera identified in the study area have been reported to be vectors of *A. marginale* (reviewed by Kocan et al., 2004). Furthermore, *A. marginale* infections were identified previously in *R. turanicus* in Sicily and in *R. bursa* and *Hy. m. marginatum* in Spain (de la Fuente et al., 2004a,b, 2005b), which suggests that these tick species may be vectors of *A. marginale* in Sicily and other regions of Europe.

The cattle herd of the farm Impastato, the only farm in which *B. annulatus* was collected, had a higher ($\chi^2 = 12.97$, d.f. = 1, $P < 0.05$) prevalence of *A. marginale* infection (100 ± 0, $n = 12$) when compared to the rest of the farms in which this tick species was not found (46 ± 8, $n = 148$). This result may suggests that *B. annulatus*, like other *Rhipicephalus (Boophilus)* species in Africa and Latin America (reviewed by Kocan et al., 2004), is a vector for *A. marginale* in Sicily. However, mechanical transmission of *A. marginale* could also play an important role in the epidemiology of bovine anaplasmosis in these regions.

Four different *msp4* genotypes were identified in *A. marginale* strains characterized in cattle farms in the Province of Palermo (Table 1). The genotypes I and II represented 83.6% and 12.7% of all *msp4* genotypes identified respectively (Table 1). Genotypes III and IV were found only once

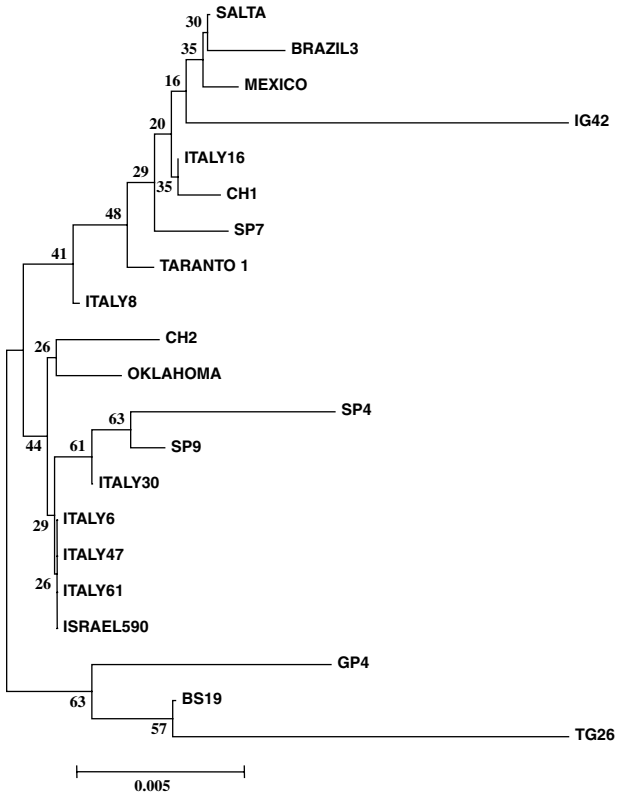

Fig. 1. Phylogenetic analysis of *Anaplasma marginale* strains based on the *msp4* gene sequences using neighbour-joining with Kimura two parameters correction and bootstrap analysis of 1000 replicates. Numbers on branches indicate per cent support for each clade. Strains Mexico (Mexico, AF428083), Brazil 3 (Brazil, AY283189), Salta (Argentina, AF428086), Oklahoma (USA, AY010252) and Israel 590 (Israel, AY786993) were used for comparison with European strains Italy 6-Italy 61 (Italy, AY702917–AY702922), Taranto 1 (Italy, AY829458), CH1-CH2 (Switzerland, AY851150), SP4-SP9 (Spain, AY456001–AY456003), and the strains identified in this work, IG42 (genotype I, DQ000620), BS19 (genotype II, DQ000618), TG26 (genotype III, DQ000621) and GP4 (genotype IV, DQ000619).

(Table 1). Genetic heterogeneity of *A. marginale* strains appears to be common in endemic areas, independent of the geographical location and predominant tick vector as was demonstrated previously in studies of *A. marginale* strains from the USA, Latin-America and Europe (reviewed by de la Fuente et al., 2005a).

Phylogenetic analysis of *msp4* sequences of European *A. marginale* strains did not provide phylogeographical information (Fig. 1). The only clade with some (63%) support was composed of strains BS19, TG26 and GP4, corresponding to genotypes II–IV identified in this study (Fig. 1). As we have demonstrated in previous studies, *msp4* sequences provided phylogeographical patterns for *A. marginale* strains from the New World (de la Fuente et al., 2002, 2003b). However, as demonstrated in this study, the inclusion of European strains in the analysis did not provide phylogeographical information and suggested the possibility of different origins for *A. marginale* in Sicily (de la Fuente et al., 2004a, 2005a). African strains of *A. marginale* should be included in the analysis to fully address the phylogenetic placement of Sicilian strains.

Characterization of the diversity of *A. marginale* strains and the correlation of infection prevalence with tick vectors reported herein is fundamental to designing epidemiological studies and control strategies for *A. marginale*. These results demonstrated the need for anaplasmosis control measures in Sicily that takes into consideration farm husbandry systems and vaccines that are protective against genetically heterogeneous populations of *A. marginale*.

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