Parasitol Res (1999) 85: 243–245

SHORT COMMUNICATION

J. Martín-Sánchez · J. Viseras · F.J. Adroher
P. García-Fernández

Nested polymerase chain reaction for detection of *Theileria annulata* and comparison with conventional diagnostic techniques: its use in epidemiology studies

Received: 30 June 1998 / Accepted: 15 August 1998

Abstract In this work we studied the ability of a nested polymerase chain reaction (PCR) to detect *Theileria annulata*, the causative agent of Mediterranean theileriosis, in blood samples obtained from cattle on farms in different Spanish regions and its possible use in epidemiology studies. Of the 214 samples analyzed, 78.04%, 69.86%, and 62.26% were found to be positive by nested PCR, indirect immunofluorescent antibody test, and optical microscopy of Giemsa-stained smears, respectively. The three techniques were in agreement in 68.6% of the results. The observation that the prevalence of Mediterranean theileriosis estimated using nested PCR alone (70.3%) and that obtained using all three diagnostic techniques together (80.4%) did not significantly differ verifies the utility of this technique in epidemiology studies.

Tropical or Mediterranean theileriosis (MT) is a disease that affects bovine cattle within a wide geographic area extending from the Mediterranean littoral regions of Europe and Africa, the Near and Middle East, and part of the former Soviet Union to India and China (Dolan 1989). In epidemiology studies, diagnosis is usually made by direct visualization with an optical microscope of the parasite in Giemsa-stained samples (OM) and by serology tests such as the indirect immunofluorescent antibody test (IFAT). Among the drawbacks of these techniques is the low sensitivity of OM, especially in the case of low levels of parasitemia, a common situation when the previously infected animals later become carriers of the parasites. For the IFAT it has been reported that the antibodies tend to disappear in long-term carriers in spite of the persistence of piroplasms, and the infection can be confirmed by xenodiagnosis (Bishop et al. 1992; Dolan 1986).

Polymerase chain reaction (PCR) offers important advantages such as the greater sensitivity and specificity over conventional techniques that has been verified in a number of studies performed on a wide range of parasites (Andersen et al. 1996; Bishop et al. 1992; Tahar et al. 1997).

The aim of this work was to examine the feasibility of using the PCR in epidemiology studies of MT and of its application in Spain. Recently, D'Oliveira et al. (1995) have described a PCR technique that specifically amplifies DNA of *Theileria annulata*. These authors use cDNA probe hybridization (*Tams*-1 gene encoding the 30-kDa major merozoite surface antigen of *T. annulata*) to confirm the amplified product and increase the sensitivity. Since we did not have the cDNA probe, we carried out a second PCR using internal primers derived from the nucleotide sequence of the *Tams*-1 gene (Shiels et al. 1994, 1995).

A total of 214 blood samples obtained from cattle on farms in the Alicante, Badajoz, Cádiz, Córdoba, Granada, and Sevilla provinces in the South of Spain were tested for the presence of *T. annulata* using three diagnostic techniques: nested PCR, IFAT, and OM. All 3 diagnostic techniques were used on 207 of the 214 samples, whereas 5 were processed by nested PCR and IFAT and the 2 remaining samples were tested by nested PCR and OM.

Of the 214 samples analyzed, 26 were obtained from fighting bulls taken to Granada to be used in bullfights and the other 188 were obtained from dairy cattle of several breeds. Of these, 79 samples consisted of thin blood smears that had been maintained at room temperature for several years (from sampling in 1993, 1994, and 1996). The remaining 135 samples consisted of

J. Martín-Sánchez · J. Viseras · P. García-Fernández
Departamento de Producción Animal, CIFA,
E-18071 Granada, Spain

J. Martín-Sánchez (✉) · J. Viseras · F.J. Adroher
Departamento de Parasitología, Facultad de Farmacia,
Campus Universitario de Cartuja,
E-18071 Granada, Spain
E-mail: fparasi@ucartuja.ugr.es,
Tel.: +34-958-243857, Fax: +34-958-243862

blood samples collected in test tubes with ethylene diaminetetraacetic acid (EDTA)-K_3 that either were immediately used for DNA extraction or had been conserved at −20 °C until processing; all of these had been collected during 1997 except for 12, which had been conserved since 1991.

Four oligonucleotide PCR primers specific for *T. annulata* were used for the nested PCR. Primers were derived from the *Tams1*-1 gene encoding the 30-kDa major *T. annulata* merozoite surface antigen (D'Oliveira et al. 1995; Shiels et al. 1994, 1995). The primers N516 and N517 were used in the first amplification reaction under the conditions described by D'Oliveira et al. (1995). The primers Ta 300: 5′CACCTCAACATACCCC3′ and Ta 750: 5′TGACCCACTTATCGTCC3′ (selected using the PRIME program of software from the Genetic Computer Group) were used in the second PCR. The final reaction volume was 50 µl and contained 16 m*M* $(NH_4)_2SO_4$, 67 m*M* TRIS-HCl (pH 8.8), 0.01% Tween 20, 1 m*M* $MgCl_2$, 200 µ*M* of each deoxynucleoside triphosphate, 20 ng of each primer, and 1 µl of amplified DNA in the first PCR. The amplification conditions included 40 cycles at 94 °C/50 s, 60 °C/30 s, and 72 °C/50 s followed by final extension at 72 °C for 5 min. The final PCR products were resolved by 1% agarose gel electrophoresis with TBE buffer (0.9 *M* TRIS, 0.9 *M* boric acid, 20 m*M* EDTA). A visible band at 721 bp in the first PCR or 453 bp in the second PCR was considered a positive result (D'Oliveira et al. 1995; the nucleotide sequence of *Tams1*-1 is available in the EMBL and GenBank data bases under the accession number U22887). Repeat amplifications with internal primer pairs verified that the amplified product was derived from the targeted *T. annulata* gene (results not shown).

Microscope examination of the Giemsa-stained smears and the serology study with the IFAT were performed by the usual methods (Burridge et al. 1974; Callow and Pepper 1974; García-Fernández et al. 1996; Viseras et al. 1997). Results were analyzed statistically using the χ^2 test.

The results obtained when all three diagnostic techniques were used are recorded in Table 1. In all, 78.04% of the samples analyzed were found to be positive using the nested PCR, 68.86% were positive in the IFAT, and 62.26% were positive in OM. Significant differences were found between the positivities obtained by OM and those obtained with the nested PCR ($P < 0.01$). In contrast, the differences observed between the positivities obtained by OM and the IFAT were not significant ($P > 0.1$). Moreover, there was no significant difference between the number of positive results obtained with the IFAT and the number obtained with the nested PCR ($P > 0.1$).

The nested PCR was more sensitive for the detection of the parasite than was either OM or the serology technique of indirect immunofluorescence; when the nested PCR served as the definition of infection the sensitivity of the IFAT was 80.1% and that of OM was 78.3%. When microscopy served as the definition of infection the sensitivity of the nested PCR was 98.4% and that of the IFAT was 89.6%. The three techniques were in agreement in 68.6% of the results. All the samples in which piroplasms were observed by OM (132) also gave a positive result in the nested PCR, except for 3 blood-smear samples; in one case, repetition of the DNA preparation process using a smear prepared with a larger blood volume followed by repetition of the double amplification yielded positive results and the false negative was attributed to insufficient sample. Repetition of this procedure was not possible in the other two cases. The IFAT results were not in agreement with those obtained with the other 2 techniques in 28 samples (see Table 1). Of these, the 13 animals with a positive IFAT and negative PCR and smear presented antibody titers of 40 (10 animals), 80 (1), and 640 (2). One animal with an antibody titer of 640 was in clinical remission after having received specific treatment for MT and could be considered cured of the parasite. Animals with a negative IFAT and positive nested PCR and OM presented levels of parasitemia lower than 0.3%.

Table 1 Results obtained using all three diagnostic techniques: nested PCR, IFAT, and OM of Giemsa-stained smears

	Nested$^+$	Nested$^-$	Total
OM$^+$ IFAT$^+$	111	2	113
OM$^+$ IFAT$^-$	15	0	15
OM$^-$ IFAT$^+$	18	13	31
OM$^-$ IFAT$^-$	17	31	48
Total	161	46	207

The use of blood smears to obtain DNA for PCR amplification has been shown to be valid, provided that a sufficient amount of blood is used for the smear. Advantages of this method include easy transport and durability of the sample, since this technique can be used on samples that have been conserved for several years.

In 46 of the blood samples analyzed, *Anaplasma* spp. were detected by OM, and 34 samples were diagnosed as being coinfected with *T. annulata* and *Anaplasma* spp. Of the remaining 12, considered as negative for *T. annulata* by OM, 7 had a positive nested PCR (specific for *T. annulata*), and the latter test was negative in the remaining 5 cases. The 7 samples with a positive nested PCR also tested as positive in the IFAT, which once again confirms the specificity of the technique.

For calculation of the prevalence of MT, only samples randomly collected in the sampling period 1996–1997 were used. These consisted of 148 samples from the regions of Cádiz (26), Córdoba (78), and Granada (44). When we considered a result to be positive when at least one of the techniques gave a positive result, the mean value of the prevalence in the study area was 80.4%. In contrast, the prevalence values obtained when each

technique was considered separately were 56.2% for OM, 66.9% for the IFAT, and 70.3% for the nested PCR. There was no significant difference between the prevalence values obtained when the nested PCR ($P > 0.1$) and the IFAT ($P > 0.05$) were used independently and the value obtained when the three techniques were used together. However, when the sole diagnostic technique used was microscopic observation of the smear, significant differences were obtained ($P < 0.001$). The percentages of positivity reported by other authors using the IFAT and OM on samples from different Spanish regions reach values of 76.9% and even 90% (Brandau et al. 1989; García-Fernández et al. 1996), although in other studies using only one technique these values are considerably lower (García-Fernández et al. 1985, 1987; Habela et al. 1993). In accordance with these authors and taking into account that we used an additional and more sensitive diagnostic technique, we would have expected to obtain higher percentages of positivity. We consider this discrepancy to be due to the different epidemiological characteristics of the provinces included in our study. The Granada province has been considered by several authors as a hypoendemic zone with a seroprevalence of 13.71% (Habela et al. 1993), whereas the Cádiz province is considered to be a hyperendemic zone (García-Fernández et al. 1985, 1987). In our case the prevalence values obtained in the provinces using the nested PCR technique ranged from 100% in Cádiz to 31.8% in Granada.

References

Andersen K, Gaafar A, El Hassan AM, Ismail A, Dafalla M, Theander TG, Kharazmi A (1996) Evaluation of the polymerase chain reaction in the diagnosis of cutaneous leishmaniasis due to *Leishmania major*: a comparison with direct microscopy of smears and sections from lesions. Trans R Soc Trop Med Hyg 90: 133–135

Bishop R, Sohanpal B, Kariuki DP, Young AS, Nene V, Baylis H, Allsopp BA, Spooner P, Dolan TT, Morzaria SP (1992) Detection of a carrier state in *Theileria parva* infected cattle by the polymerase chain reaction. Parasitology 104: 215–232

Brandau C, Monge A, Fermín ML, Jiménez-Mazzucchelli F, Tesouro MA (1989) Tratamiento de la theileriosis bovina con parvacuona y buparvacuona; control y seguimiento. Vet Rec [Spanish edn]: 279–281

Burridge MJ, Brown CGD, Kimber CD (1974) *Theileria annulata*: cross reactions between a cell culture schizont antigen and antigens of East African *Theileria* species in the indirect fluorescent antibody test. Exp Parasitol 35: 374–379

Callow LL, Pepper PM (1974) Measurement of and correlations between fever, changes in the packed cell volume and parasitaemia infection with *Babesia argentina*. Aust Vet J 50: 1–5

Dolan TT (1986) Chemotherapy of East Coast fever: the long term weight changes, carrier state and disease manifestations of parvaquone treated cattle. J Comp Pathol 96: 137–146

Dolan TT (1989) Theileriosis: a comprehensive review. Rev Sci Tech Off Int Epizoot 8: 11–36

D'Oliveira C, Weide M, Habela MA, Jacquiet P, Jongejan F (1995) Detection of *Theileria annulata* in blood samples of carrier cattle by PCR. J Clin Microbiol 33: 2665–2669

García-Fernández P, Romero-Rodríguez J, Hueli LE (1985) Piroplasmosis bovinas en Andalucía. I. Estudio en reses procedentes de mataderos. Rev Iber Parasitol 45: 49–58

García-Fernández P, Romero-Rodríguez J, Hueli LE (1987) Piroplasmosis bovinas en Andalucía. II. Estudio en explotaciones agropecuarias. Rev Iber Parasitol 47: 7–13

García-Fernández P, Hueli LE, Viseras-Alarcón J (1996) Encuesta epizootiológica de la theileriosis bovina por *Theileria annulata* en el toro de lidia. Med Vet 13: 538–542

Habela M, Aranda C, Rol JA, Navarrete I (1993) Seroprevalencia de la theileriosis Mediterránea en ganado bovino de aptitud lechera en la provincia de Granada (España). Acta Parasitol Port 1: 247

Shiels BR, Smyth A, Dickson J, McKellar S, Tetley L, Fujisaki D, Hutchinson B, Hinnaird JH (1994) A stoichiometric model of stage differentiation in the protozoan parasite *Theileria annulata*. Mol Cell Differ 2: 101–125

Shiels BR, D'Oliveira C, McKellar S, Ben-Miled L, Kawazu S, Hide G (1995) Selection of diversity at putative glycosylation sites in the immunodominant merozoite/piroplasm surface antigen of *Theileria* parasites. Mol Biochem Parasitol 72: 149–162

Tahar R, Ringwald P, Basco LK (1997) Diagnosis of *Plasmodium malariae* infection by the polymerase chain reaction. Trans R Soc Trop Med Hyg 91: 410–411

Viseras J, García-Fernández P, Adroher FJ (1997) Isolation and establishment in in vitro culture of a *Theileria annulata*-infected cell line from Spain. Parasitol Res 83: 394–396