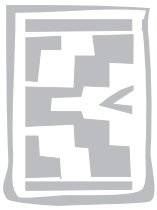

The pCS20 PCR assay for *Ehrlichia ruminantium* does not cross-react with the novel deer ehrlichial agent found in white-tailed deer in the United States of America

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ABSTRACT

MAHAN, S.M., SIMBI, B.H. & BURRIDGE, M.J. 2004. The pCS20 PCR assay for *Ehrlichia ruminantium* does not cross-react with the novel deer ehrlichial agent found in white-tailed deer in the United States of America. *Onderstepoort Journal of Veterinary Research*, 71:99–105

White-tailed deer are susceptible to heartwater (*Ehrlichia* [*Cowdria*] *ruminantium* infection) and are likely to suffer high mortality if the disease spreads to the United States. It is vital, therefore, to validate a highly specific and sensitive detection method for *E. ruminantium* infection that can be reliably used in testing white-tailed deer, which are reservoirs of antigenically or genetically related agents such as *Ehrlichia chaffeensis*, *Anaplasma (Ehrlichia) phagocytophilum* (HGE agent) and *Ehrlichia ewingii*. Recently, a novel but as yet unnamed ehrlichial species, the white-tailed deer ehrlichia (WTDE), has been discovered in deer populations in the United States. Although the significance of WTDE as a pathogen is unknown at present, it can be distinguished from other *Ehrlichia* spp. based on 16S rRNA gene sequence analysis. In this study it was differentiated from *E. ruminantium* by the use of the pCS20 PCR assay which has high specificity and sensitivity for the detection of *E. ruminantium*. This assay did not amplify DNA from the WTDE DNA samples isolated from deer resident in Florida, Georgia and Missouri, but amplified the specific 279 bp fragment from *E. ruminantium* DNA. The specificity of the pCS20 PCR assay for *E. ruminantium* was confirmed by Southern hybridization. Similarly, the 16S PCR primers (nested) that amplify a specific 405–412 bp fragment from the WTDE DNA samples, did not amplify any product from *E. ruminantium* DNA. This result demonstrates that it would be possible to differentiate between *E. ruminantium* and the novel WTDE agent found in white tailed deer by applying the two respective PCR assays followed by Southern hybridizations. Since the pCS20 PCR assay also does not amplify any DNA products from *E. chaffeensis* or *Ehrlichia canis* DNA, it is therefore the method of choice for the detection of *E. ruminantium* in these deer and other animal hosts.

Keywords: *Ehrlichia (Cowdria) ruminantium*, PCR, pCS20 sequence, white-tailed deer, white-tailed deer ehrlichia

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INTRODUCTION

Heartwater is an acute infectious disease caused by *Ehrlichia (Cowdria) ruminantium* and is transmitted to domestic and wild ruminants by *Amblyomma* ticks (Walker & Olwage 1987). In susceptible domestic ruminants, heartwater can cause severe losses through high mortality rates of up to 90 % (Mahan, Smith, Kumbula, Burridge & Barbet 2001). Animals which recover from primary infection become asymptomatic carriers of *E. ruminantium* and serve as reservoirs of infection for ticks (Andrew

& Norval 1989; Peter, Burridge & Mahan 2002). Several wild game species are affected by *E. ruminantium*. Some wild animal species are refractory to *E. ruminantium* infection, while some serve as carriers and others, such as white-tailed deer (*Odocoileus virginianus)*, springbok (*Antidorcas marsupialis)* and kafue lechwe (*Kobus leche kafuensis)* suffer high mortality when infected experimentally or naturally (Camus, Barré, Martinez & Uilenberg 1996; Peter *et al.* 2002). The role played by wild animal species in the epidemiology of heartwater is still not fully understood. Nevertheless, it has been shown that *E. ruminantium* can be maintained in *Amblyomma hebraeum* ticks found exclusively in the Kruger National Park in South Africa where there has been no contact between the wild game species and cattle for at least 40 years (Peter, Bryson, Perry, O'Callaghan, Medley, Smith**,** Mlambo, Horak, Burridge & Mahan 1999), indicating that the ticks maintain their infection by feeding on infected wild animal species found in the Park.

In the United States of America (USA), the white-tailed deer population plays a key role in the epidemiology of ehrlichial infections which cause serious illnesses in humans (Dawson, Stallknecht, Howerth, Warner, Biggie, Davidson, Lockhart, Nettles, Olson & Childs 1994; Ewing, Dawson, Kocan, Barker, Warner, Panciera, Fox, Kocan & Blouin 1995; Lockhart, Davidson, Stallknecht, Dawson & Howerth 1997). White-tailed deer are reservoirs of *Ehrlichia chaffeensis* the causative agent of human monocytic ehrlichiosis, *Anaplasma (Ehrlichia) phagocytophilum* (Dawson, Warner, Baker, Ewing, Stallknecht, Davidson, Kocan, Lockhart & Olson, 1996), the cause of human granulocytic ehrlichiosis (previously known as the HGE agent), *Ehrlichia ewingii,* one of the causative agents of canine granulocytotropic ehrlichiosis and also causes infections in humans (Yabsley, Varela, Tate, Dugan, Stallknecht, Little & Davidson 2002) and a novel ehrlichial organism, white-tailed deer ehrlichia (WTDE). The WTDE which is distinct from the other ehrlichial agents based on 16S rRNA sequences (Dawson *et al.* 1996; Brandsma, Little, Lockhart, Davidson, Stallknecht & Dawson 1999), is closely related to *Anaplasma (Ehrlichia) phagocytophila* genogroup, which also includes *Ehrlichia equi, Ehrlichia platys* and the HGE agent, also based on 16S analysis (Little, Dawson, Lockhart, Stallknecht, Warner & Davidson 1997). Heartwater, at present, does not occur in the USA but if it did invade the country, white-tailed deer would be a susceptible target population. It is, therefore, important to differentiate infections caused by the various ehrlichial agents. Serological assays available for detection of antibodies to *E. ruminantium* also detect cross-reactions due to other *Ehrlichiae* hence compromising interpretations of serological results (Mahan, Barbet, Tebele, Nyathi, Wassink, Semu, Peter & Kelly 1993). Consequently, it is critical to have an assay that can be used with confidence for the detection and differentiation of *E. ruminantium* infection from infections caused by other agents. The pCS20 PCR assay has been shown to be highly specific and sensitive for detection of *E. ruminantium* DNA in ticks and in clinically ill animals (Mahan, Waghela, McGuire, Rurangirwa, Wassink & Barbet 1992; Mahan *et al*. 1993; Peter, Deem, Barbet, Norval, Simbi, Kelly & Mahan 1995; Allsopp, Hattingh, Vogel & Allsopp 1999; Peter, Barbet, Alleman, Simbi, Burridge & Mahan 2000). In addition, it has previously been demonstrated that the pCS20 PCR assay does not amplify any DNA products from *E. chaffeensis* and *Ehrlichia canis* DNA (Peter *et al*. 1995, 2000), the infectious agents most closely related to *E. ruminantium* by 16S rDNA analysis and by analysis of genes encoding the outer membrane proteins (Reddy, Sulsona, Barbet, Mahan, Burridge & Alleman 1998; Sulsona, Mahan & Barbet 1999; Yu, McBride, Zhang & Walker 2000; Ohashi, Rikihisa & Unver 2001). The pCS20 PCR assay was used to differentiate between the WTDE agent and *E. ruminantium*.

MATERIALS AND METHODS

White-tailed deer ehrlichia and *E. ruminantium* DNA

Blood samples from white-tailed deer infected with the WTDE were collected from deer located in Georgia, Missouri and Florida in USA, and DNA was prepared as described previously (Little *et al.* 1997). These DNA samples were a kind donation from Dr Susan Little of University of Georgia. *Ehrlichia ruminantium* DNA was extracted from bovine endothelial cell cultures infected with the Crystal Springs strain (Byrom, Yunker, Donovan & Smith 1991). Briefly, infected bovine endothelial cell cultures were spun at low speed (400 x *g*) to pellet large cellular material; this was followed by high-speed centrifugation (30 000 x *g*) of the supernatants to pellet the organisms. The organisms were resuspended in phosphate buffered saline (PBS) and purified through discontinuous Percol gradients ranging from 0–40 % in PBS to separate them from bovine cellular materials at 400 x *g*. The purified organisms were harvested from the top

layer (0 %) and washed twice with PBS by centrifugation at 30 000 x *g* and thereafter resuspended in fresh PBS. DNA was extracted from the organisms by the Qiagen technique as recommended by the manufacturers (Qiagen Genomic DNA Extraction kit, Qiagen Corp, Valencia. CA).

PCR assay to detect white-tailed deer ehrlichia DNA

A diagnostic nested PCR was performed using an established protocol (Little *et al.* 1997). The PCR primers were kindly provided by Dr Susan Little. A primary reaction using external general *Ehrlichia* 16S primers ECB and ECC on white-tailed deer or *E. ruminantium* DNA was conducted as previously described (Little *et al.* 1997). A secondary reaction on the primary PCR product was carried out using internal primers DGA and GA1UR specific to the WTDE (Little *et al*. 1997). For the primary amplification, a 50 µl reaction mixture containing 5 µℓ of DNA, 10 mM Tris Cl (pH 8.3), 50 mM KCl, 0.001 % gelatine, 2.5 mM $MgCl_2$, 200 µm each of deoxynucleoside triphosphates dATP, dCTP, dGTP and dTTP, 1.25 U *Taq* DNA polymerase (Perkin Elmer Corporation, Norwalk, Connecticut, USA), and 1 µM each of primers ECB (5'-CGTATTACCGCGGCTGCTGGCA-3'), and ECC (5'-AGAACGAACGCTGGCGGCAAGCC-3'), was overlaid with 50 µℓ of mineral oil (Sigma Co., St Louis, Missouri, USA). Amplification was done by denaturation at 94 °C for 1 min, annealing at 45 °C for 2 min and extension at 72 °C for 2 min. A total of 40 cycles were performed followed by an automatic extension for 10 min at 72 °C. The secondary nested PCR was performed using 1 µℓ of the product from the primary reaction mixed as above but with 5 µm each of the primers DGA 5'-TTATCTCTGTAGCTTGCTACG-3' and GAIUR 5'-GAGTTTGCCGGGACTTCTTCT-3'. The reaction was amplified for 30 cycles of 1 min denaturation at 94 °C, 2 min annealing at 55 °C and 2 min extension at 72 °C. The products from the second nested PCR reactions were analyzed by electrophoresis on a 1.5 % agarose gel and viewed with UV light illumination and photography after staining with ethidium bromide. The PCR products were transferred onto a nylon membrane (NEN Life Science Products, Boston, Massachusetts) and hybridized with a ^{32}P-labelled specific pCS20 DNA probe or the 16S WTDE amplified DNA fragment as probe, as described previously (Mahan *et al.* 1992; Peter *et al.* 2000). The results of the hybridizations were evaluated by autoradiography. In all the PCR assays, a reagent blank control (no template DNA) and a respective positive control (WTDE or *E. ruminantium* DNA) reaction were included.

The *E. ruminantium*-specific pCS20 PCR assay

A nested PCR, specific for *E. ruminantium*, was also performed to amplify DNA from *E. ruminantium* and WTDE-infected deer DNA samples. In a primary reaction, primers U24 (5'-TTTCCCTATGATACAGAAGGTAAC-3') and L24 (5'-AAAGCAAGGATTGTGATCTGGACC-3') were used to amplify from *E. ruminantium* DNA (350 bp DNA fragment) and from WTDE DNA. In the second or nested PCR assay with primers AB 128 (5'-ACTAGTAGAAATTGCACAATCTAT-3') and AB129 (5'-TGATAACTTGGTGCGGGAAATCCTT-3'), a 279 bp *E. ruminantium*-specific DNA fragment was amplified as described previously (Mahan *et al.* 1992; Peter *et al.* 2000). Each PCR reaction contained a volume of 50 µℓ containing the following components: 5 µℓ of template DNA, 10 mM Tris pH 8.3, 50 mM KCl, 0.001 % gelatine, 3.0 mM $MgCl_2$, 0.5 µm each of the primers L24 and U24 (Genosys Biotechnologies, Inc. The Woodlands, Texas, USA), 200 µm of deoxynucleoside triphosphates dATP, dCTP, dGTP and dTTP, and 1.25 U *Taq* polymerase. Denaturation of DNA was done at 94 °C for 1 min, annealing of primers was at 55 °C for 1 min and extension at 72 °C for 2 min. After 45 cycles, an automatic 10 min extension at 72 °C was followed by a soak at 4 °C. In the second or nested reaction, 1 µℓ of the primary product was used in a 50 µℓ reaction mix with the same reaction and amplification conditions as the primary reaction but with the primers: AB 128 and AB 129. In all the PCR reactions, a reagent blank control (no template DNA) and a respective positive control was included. The products from the second nested PCR reactions were analyzed by electrophoresis on a 1.5 % agarose gel and viewed with UV light illumination and photography after staining with ethidium bromide. The PCR products were transferred onto nylon membrane (NEN Life Science Products, Boston. MA) and hybridized with a ^{32}P-labelled specific pCS20 DNA probe or the 16S WTDE-amplified DNA fragment as probe, as described above. The results of the hybridizations were evaluated by autoradiography.

RESULTS

The nested WTDE 16S specific primers DGA and GAIUR amplified a DNA product of 405–412 bp

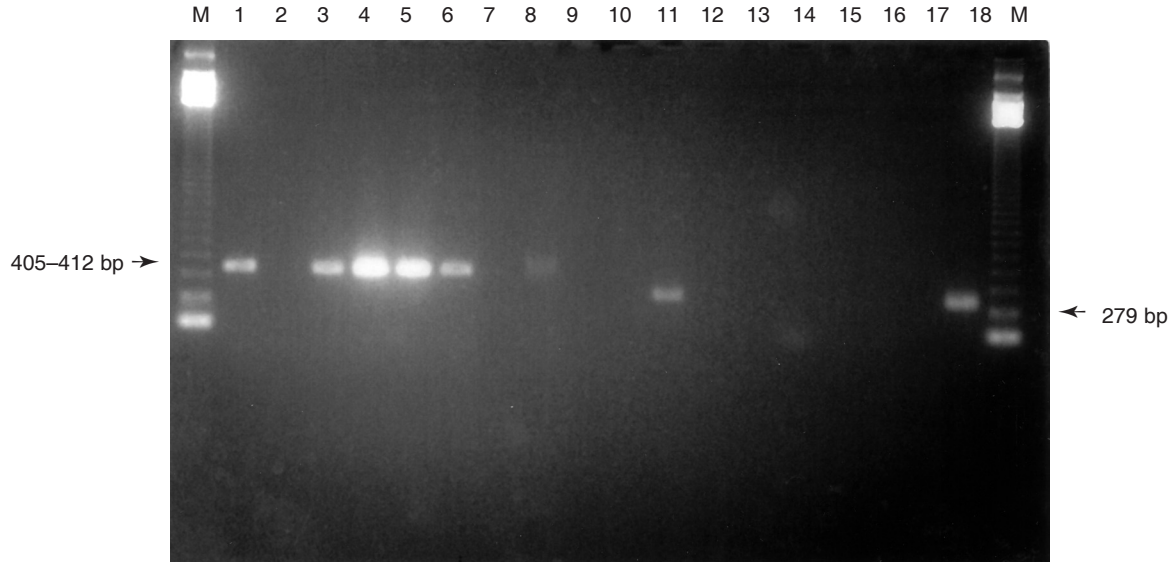

FIG. 1 A pCS20 PCR assay for *E. ruminantium* does not detect DNA from white tailed deer *Ehrlichia*. Agarose gel electrophoresis of PCR products with primers specific for WTDE show amplification of a DNA product of 405–412 bp from WTDE DNA (lanes 1, 3, 4, 5 and 6) but not from *E. ruminantium* Crystal Springs strain DNA (lane 8). Lanes 11 and 18 represent the results of the *E. ruminantium*-specific PCR assay. No amplification was detected when the pCS20 PCR was done on WTDE DNA (lanes 13–16) but amplification of a 279 bp fragment was seen with *E. ruminantium* DNA (lanes 11 and 18). Lane M is the 123 bp DNA markers. WTDE DNA samples are from white tailed deer from the following locations: lanes 1 and 6 are OS 82 (Ossabaw Island Georgia), lane 3 is MO (Missouri), lane 4 is Florida 2 and lane 5 is Florida 1. Lanes 2 and 17 are reagent blanks and 7, 9, 10, 12 and 17 are empty

from the deer samples containing WTDE DNA from Florida, Georgia and Missouri, but not from *E. ruminantium* DNA (Fig. 1). The general *Ehrlichia* primers ECB and ECC amplified a product from the *E. ruminantium* DNA which was of higher molecular weight than the product from the nested PCR on WTDE DNA, and appeared as a diffuse band when visualized by agar gel electrophoresis following the nested PCR (Fig. 1, lane 8). In comparison, the (nested) primers AB 128 and AB 129 specific for the pCS20 sequence of *E. ruminantium*, amplified the 279 bp fragment from *E. ruminantium* DNA but not from the WTDE-infected deer DNA samples originating from Florida, Georgia and Missouri (Fig. 1). Further confirmation of the specificity of the two PCR reactions was done by southern hybridization. The labelled pCS20 probe of *E. ruminantium* hybridized only to the products from the pCS20 PCR assays on *E. ruminantium* DNA and not to any of the samples which were amplified from WTDE DNA. The 405–412 bp probe of WTDE hybridized only to the products from the nested PCR done with the WTDE primers on WTDE DNA and not to products from *E. ruminantium* DNA (Fig. 2A and B).

DISCUSSION

White-tailed deer play an important role in the epidemiology of infections which affect humans, as they are reservoirs of *E. chaffeensis, E. ewingii* and *Anaplasma (Ehrlichia) phagocytophilum* (the HGE agent) (Little, Stallknecht, Lockhart, Dawson & Davidson 1998). The role, if any, of the recently identified WTDE in the pathogenesis of disease remains to be determined because, it has not yet been isolated and characterized *in-vitro*. However, based on the 16S sequences, the WTDE has been shown to be unique and different from other ehrlichial agents so far characterized, but it is closely allied to *E. phagocytophila* genogroup, which includes *E. platys, E. equi* and *Anaplasma (Ehrlichia) phagocytophilum* (the HGE agent) (Dawson *et al*. 1996).

The phylogenetic classification of the *Ehrlichia* and *Rickettsia* spp. has been recently revised based on sequence similarities of the16S gene, the citrate synthase gene, the GroEL sequences and genes encoding structural outer membrane proteins of these organisms that were previously classified

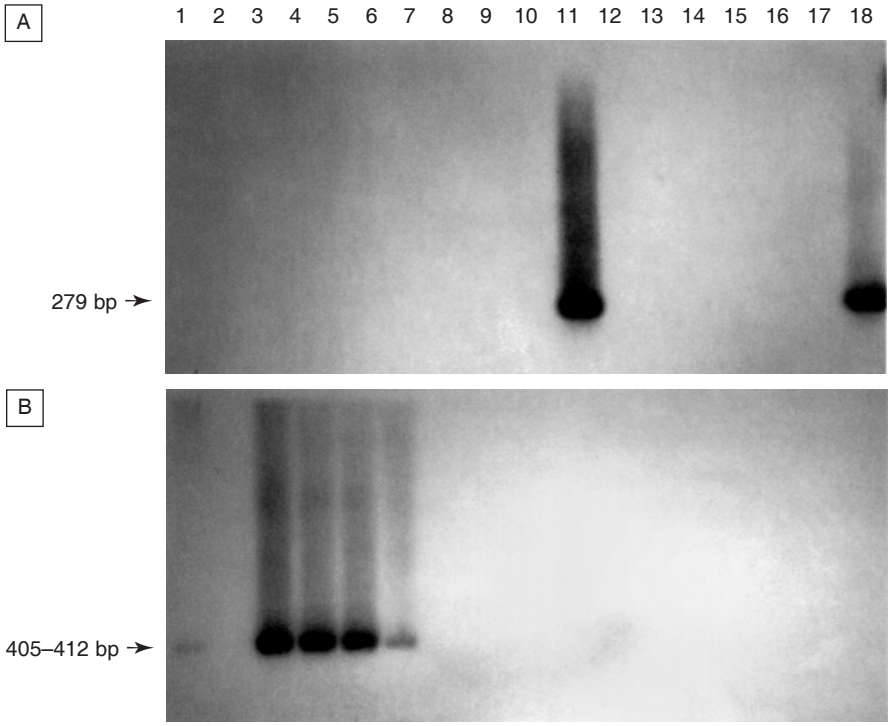

FIG. 2A Southern Hybridization of pCS20 ^{32}P-labelled probe with the PCR products as in Fig. 1, to show positive hybridization of the pCS20 *E. ruminantium* probe to the 279 bp products from *E. ruminantium* DNA. No hybridization occurred with the WTDE DNA samples

FIG. 2B Southern Hybridization of 405–412 bp WTDE PCR product ^{32}P-labelled as probe with the PCR products as in Fig. 1 and show positive hybridization of the probe to the WTDE DNA samples but not to the samples from *E. ruminantium* DNA

under different Families or Tribes (Dumler, Barbet, Bekker, Dasch, Jongejan, Palmer, Ray, Rikihisa & Rurangirwa 2001). Due to its genetic similarity to *E. chaffeensis* and *E. canis*, *Cowdria ruminantium* has been placed in the genus *Ehrlichia* and renamed *Ehrlichia ruminantium*. This genus now includes *E. ruminantium*, *E. chaffeensis, E. canis, E. ewingii,* and *Ehrlichia muris*. Because of the close genetic and structural relationship of these *Ehrlichia* spp., and their related epidemiology, it is important to differentiate them from each other using reliable detection assays. Serological assays are unable to differentiate definitively between *E. ruminantium* and several of the related ehrlichial agents because of marked cross-reactions between immunogenic proteins of these organisms. The PCR assay has proved to be more specific and sensitive in differentiating these related organisms. The pCS20 PCR assay, which is specific for *E. ruminantium*, is the method of choice for its detection and differentiation from the other ehrlichial agents (Peter *et al.* 1995, 2000; Allsopp *et al.* 1999). It was also able to differentiate between the WTDE organism and *E. ruminantium* in this study. This was shown by both agarose gel electrophoresis and Southern hybridizations. The nested PCR assay for the WTDE was also found to be specific. However, the fragment that was amplified after the primary PCR using ECB and ECC primers on *E. ruminantium* might be confused as being a true amplification, and hence Southern hybridization with the 405–412 bp fragment of WTDE DNA as probe would assist in determining whether the agent being detected is *E. ruminantium* or the WTDE agent. The pCS20 PCR assay would be the method of choice for the detection of *E. ruminantium* infections in the white tailed deer, if heartwater did spread to the USA. This assay will also not detect infections caused by other related ehrlichial agents and, hence, can be applied reliably for surveillance of heartwater in animals and vectors.

The risks of the introduction of heartwater onto the American mainland associated with animal movements are increasing (Burridge, Simmons, Peter &

Mahan 2002). One measure to reduce these risks is the use of improved tests, such as the PCR assay, for the screening of animals to prevent the entry of carriers of *E. ruminantium*. For these tests to be used reliably, it is important that they do not produce cross-reactions with native ehrlichial parasites. This report demonstrates that the pCS20 PCR assay for *E. ruminantium* does not cross-react with the novel WTDE organism, making it a valuable tool in monitoring and prevention of introduction of heartwater into the USA and elsewhere.

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