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Detection of *Borrelia burgdorferi* DNA in Lizards from Southern Maryland

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Abstract

Lizards serve as hosts for *Ixodes* ticks in the western and southeastern United States and may affect the transmission cycles of *Borrelia burgdorferi* in these regions. In Maryland, the role of lizards in the maintenance and transmission cycle of this pathogen has not been examined. We tested 29 lizards (*Sceloporus undulatus* and *Eumeces* spp.) and 21 ticks from these lizards for the presence of *B. burgdorferi*. Eight lizards were positive by polymerase chain reaction (PCR) for at least one *B. burgdorferi*-specific marker. This is the first report of *B. burgdorferi* sensu stricto detected in lizards in the mid-Atlantic region. Although the viability of the *B. burgdorferi* in these lizards was unconfirmed, recovery of bacterial DNA in a tail snip suggests that the infections in these lizards were disseminated. This study suggests that some lizards indigenous to the mid-Atlantic region may serve as alternative reservoirs for *B. burgdorferi*. In areas where lizard densities are high, these less efficient reservoirs may affect the enzootic cycle of this tick-borne pathogen.

Keywords

Lyme disease; *Borrelia burgdorferi*; Lizards

INTRODUCTION

In the eastern United States, *Borrelia burgdorferi* sensu stricto, the causative spiro-chete of Lyme disease, is vectored by the black-legged tick, *Ixodes scapularis*, and is primarily maintained in an enzootic cycle between *I. scapularis* and the white-footed mouse, *Peromyscus leucopus*. This enzootic transmission cycle may be disrupted by the presence of alternative hosts that are less competent reservoirs than are *P. leucopus* (Oliver et al. 2003). While immature *I. scapularis* tend to feed on small mammals such as white-footed mice and Eastern chipmunks (*Tamias striatus*) that have high reservoir competence for *B. burgdorferi*,

they also feed on other mammals, birds, and lizards (Giardina et al. 2000, Keirans et al. 1996).

In the southeastern and western United States, *Ixodes* ticks involved in *B. burgdorferi* transmission parasitize several species of lizards, including *Eumeces laticeps*, *E. fasciatus*, *Sceloporus undulatus*, and *S. occidentalis* (Apperson et al. 1993, Lane and Loye 1989, Levine et al. 1997). Ticks do not parasitize lizards as frequently in northern states (Durden et al. 2002), possibly because of the greater relative abundance of rodents, or possibly because of the scarcity of lizards in these states (Apperson et al. 1993, Oliver et al. 1993). Although *Ixodes* ticks may successfully feed on several species of lizards, the impact of this feeding on the enzootic cycle of *B. burgdorferi* appears to largely depend on the lizard species involved because the ability of lizards to serve as reservoirs for this spirochete has been shown to vary between species (Lane and Quistad 1998, Levin et al. 1996). In the western United States, *I. pacificus*, the regional vector of *B. burgdorferi*, parasitizes the western fence lizard (*S. occidentalis*) even when small mammal hosts are available (Lane and Loye 1989). Lane and Quistad (1998) identified a factor in the alternative complement pathway of the western fence lizard, which not only causes the lizard to be reservoir incompetent, but also clears the *B. burgdorferi* infection in the feeding tick. This factor also occurs in the blood of the southern alligator lizard (*Elgaria multicarinata*), which is resistant to infection with *B. burgdorferi* even though it serves as a host for ticks (Kuo et al. 2000, Wright et al. 1998). *Borrelia burgdorferi* s.s. is resistant to killing by either bird or rodent complement, which may explain, at least in part, the difference in the general reservoir competence of these animals (Kurtenbach et al. 1998).

In contrast to the western fence and southern alligator lizards, which do not become infected with *B. burgdorferi*, Levin et al. (1996) determined that the southeastern five-lined skink (*Eumeces inexpectatus*) and the green anole (*Anolis carolinensis*) can acquire *B. burgdorferi* infection through both needle inoculations and tick bites. Dissemination of *B. burgdorferi* in these lizard species requires several weeks, unlike mammals that are infectious only a few days postexposure (Levin et al. 1996). Despite the relatively long period for dissemination that may be attributable to reptile biology, these lizards, when present may have the ability to serve as alternative reservoirs for *B. burgdorferi* but with lower efficiency. Lizards such as the broad-headed skink (*Eumeces laticeps*) are relatively abundant in the mid-Atlantic and southeastern United States and are not resistant to multiple feedings by *I. scapularis* (Galbe and Oliver 1992). Therefore, in the southeast, where immature *I. scapularis* frequently feed on lizards, it has been hypothesized that the prevalence of *B. burgdorferi* is depressed, in part, because of the decreased number of ticks feeding on mammalian and avian hosts considered to have higher reservoir competence (Apperson et al. 1993, Oliver et al. 1993). A recent study by Clark et al. (2005) detected *B. burgdorferi* sensu lato DNA by polymerase chain reaction (PCR) in lizards in the southeastern states of Florida and South Carolina. Because *Borrelia* DNA was obtained from tail blood, disseminated infections were presumed to have occurred in these lizards. In addition, the detected spirochete DNA was interpreted to indicate the presence of viable organisms because the lizards were captured during months that immature *I. scapularis* were not active (Clark et al. 2005). Overall, 53.8% of the lizards in that study were positive for *B. burgdorferi* sensu lato by PCR;

however, only 11.6% were positive for all amplification targets (*flaB*, *ospA*, and *p66*) (Clark et al. 2005).

Studies by Hofmeister et al. (1999) and by Anderson (2004) established that *P. leucopus* and *I. scapularis* coexist in central and southern Maryland. However, the prevalence of *B. burgdorferi* in *P. leucopus* in southern Maryland varies greatly. At least three species of lizards, *S. undulatus*, *E. laticeps*, and *E. fasciatus*, have been observed at several collection sites where previous studies of *B. burgdorferi* have been conducted. We hypothesized that these lizards could impact the enzootic transmission of *B. burgdorferi* in Maryland. To evaluate this hypothesis, the current study was designed to determine if lizards are parasitized by immature *I. scapularis* and infected with *B. burgdorferi* s.s.

MATERIALS AND METHODS

Sample collection and DNA extraction

Lizards were trapped by pitfalls, drift fences, and hand capture from May through September 2002 and May, June, and September 2003 in Charles, St. Mary's, and Queen Anne's counties, Maryland (Table 1). Ticks were removed from lizards and stored at -20°C . Tissue samples were collected by tail snip and stored on ice in the field and at -20°C in the laboratory until use. Using a sterile scalpel blade, the last 2 cm of the tail was removed and placed in a 2-mL screw-cap tube. Lizards were identified using a field guide (Behler and King 1979).

DNA was extracted from 0.5 cm of the proximal end of the tail snip using the QiAamp DNA MiniKit (Qiagen, Inc., Valencia, CA) following the manufacturer's protocol for tissue extraction with minor modifications. The proteinase K digestion was extended overnight and a second wash using each wash buffer (AW1 and AW2) was added to remove additional salts resulting from the extended digestion. After the lysis step the samples were centrifuged at 13,000 rpm for 3.5 minutes to pellet any undigested tissue. The extraction was continued using the supernatant. DNA extractions for the ticks were performed using the QiAamp DNA MiniKit, following the tissue extraction protocol. Prior to extraction, the species, developmental stage, and engorgement status of each tick was recorded.

Lizard and tick 12S PCR

Extraction fidelity from the tail snip was evaluated by PCR amplification of a fragment from the reptile mitochondrial 12S ribosomal genome. In addition, the tick extractions were analyzed for lizard 12S rDNA to determine if the blood meal could be detected in the extractions. Lizard 12S PCR primers (Table 2) were designed using Primer3 (www.frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and were expected to amplify a 298 bp fragment. Each 50- μL PCR reaction contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl_2 , 0.01% w/v gelatin, 100 μM each dNTP, 50 pmol each primer, 2.5 U *Taq* polymerase, and 1.5 μL template DNA. For PCR, an initial denaturation step at 95°C for 3 minutes was followed by 20 cycles of 95°C for 30 seconds, 40°C for 1 minute, and 72°C for 1 minute, which was then followed by 40 cycles of 95°C for 30 seconds, 50°C for 1 minute, and 72°C for 1 minute, with a final extension at 72° for 7 minutes. Tick extraction

fidelity was evaluated by PCR amplification of tick mitochondrial 12S rDNA. The PCR protocol for the 12S rDNA has been described elsewhere (Norris et al. 1996). Amplifications were completed on an MJ Research PTC-200 thermal cycler (MJ Research, Watertown, MA). Negative controls were included in all PCR runs. Amplicons were visualized with ethidium bromide on 2% agarose gels.

***Borrelia burgdorferi* PCR and sequencing**

Lizard and tick DNA extractions were screened for *B. burgdorferi* using nested PCR for four molecular markers: *flaB*, *ospA*, *ospC*, and *rrf-rrl*. All primer sequences and references are listed in Table 2. PCR for the flagellin gene (*flaB*) fragment was completed as described by Johnson et al. (1992). The *ospA* PCR for the outer surface protein A gene fragment was based on the nested PCR protocol described by Guttman et al. (1996) with the following modifications: 1.5 mM MgCl₂, 1 U of *Taq* polymerase, and 1 µL of DNA was used for both the exterior and interior reactions. The nested PCR for the outer surface protein C (*ospC*) gene fragment was based on Wang et al. (1999) and amplified the 3' end of the *ospC* fragment by using both exterior primers for the first round PCR and the interior forward and exterior reverse for the second round PCR. The only modifications were utilization of 1.5 µL of template DNA for the exterior first round reaction and an increase in the annealing temperature from 54°C to 60°C for 35 seconds in the second round of PCR to increase efficiency. The PCR protocol for the *rrf* (5S)–*rrl* (23S) intergenic spacer region (*rrf-rrl*) fragment was followed as published by Rijpkema et al. (1995). Positive and negative controls were included for all *B. burgdorferi* amplifications. All amplicons were run on 2% agarose gels and visualized with ethidium bromide. Positive samples were defined as those that produced PCR products at the expected sizes.

Positive PCR products for *flaB*, *ospA*, *ospC*, and *rrf-rrl* were sequenced directly in both directions and consensus sequences were generated using SeqMan II (DNASTAR Inc., Madison, WI) (GenBank accession numbers DQ867082–DQ867087). Consensus sequences for each gene fragment were aligned with corresponding sequences retrieved from the public domain using MultAlin (Institut National de la Recherche Agronomique, Paris, France) (Corpet 1988) and then were compared in a phylogenetic assessment for identity using PAUP version Beta 10 (Sinauer Associates, Inc. Sunderland, MA). Support for molecular identity of the *Borrelia* gene fragments recovered from lizard and tick sources was determined by bootstrap support in phylogenetic reconstructions based on Maximum Parsimony (MP) and Maximum Evolution (Neighbor Joining) models (Swofford 2003). Phylogenies were constructed using heuristic search methods, adding sequences into the initial tree randomly and using the tree bisection-reconnection (TBR) branch swapping algorithm.

RESULTS

A total of 29 lizards were trapped and sampled in 2002 and 2003 at 11 sites in Charles, St. Mary's, and Queen Anne's counties, Maryland. Fifteen of the lizards were *Sceloporus undulatus*. The remaining 14 were either *Eumeces fasciatus* (*n* = 12) or *E. laticeps* (*n* = 2) (Table 1). Tail snips were collected from all lizards.

DNA extractions from all the tail snips were successful as indicated by the lizard 12S PCR results. Overall, 27.6% ($n = 8$) of the lizards sampled tested positive for at least one *B. burgdorferi* target. One lizard sample was positive for *flaB*, two for *ospA*, 4 for *ospC*, and 3 for *rrf-rrl*. Two samples were positive for more than one marker (Table 3). These samples were positive for *rrf-rrl* and either *ospA* or *ospC*, respectively. Three of the eight positive lizards were *S. undulatus*, four were *E. fasciatus*, and one was *E. laticeps*. Five of the amplicons were confirmed to be *B. burgdorferi* s.s. by direct sequencing and 90%–99% bootstrap support in comparison to published sequences.

Ticks found on the lizards were also examined. A total of 21 ticks were removed from 11 (38%) lizards (2 from *S. undulatus*, 8 from *E. fasciatus*, 1 from *E. laticeps*). These lizards were collected at 6 of the 11 sites (NGF, WF, LSP, GW, CHR, LBR). Four (36.4%) of these lizards (*E. fasciatus* from CHR and GW) were positive for *B. burgdorferi*. Ticks were removed most frequently from the front leg region; ticks were also attached around the head. Five *E. fasciatus* and two *S. undulatus* had two or more ticks present. The presence of ticks on *E. fasciatus* was significantly higher than on the other two species of lizards ($\chi^2 = 8.19$, $p = 0.017$).

All ticks were morphologically identified as *I. scapularis*. Four (19%) of the ticks were larvae; the remaining 17 were nymphs. Of the larvae, 2 were flat (unfed), 1 was partially engorged, and 1 was engorged. Of the nymphs, 6 were flat, 7 were partially engorged, and 4 were engorged. Extractions were successful for 18 ticks based on a positive PCR product for the tick 12S mitochondrial rDNA target.

Only one tick, a flat nymph collected from an *E. fasciatus* at LSP, was positive for *B. burgdorferi* s.s. by PCR. Neither the lizard, which had four ticks attached, nor the other three ticks from the lizard were positive for any *B. burgdorferi* targets. As a result of negligible rates of transovarial transmission of this pathogen, we would expect *B. burgdorferi* detected was acquired through feeding as a larval tick on a previous host.

DISCUSSION

The role that lizards play in the transmission cycle of *B. burgdorferi* in the United States varies regionally, depending on the species of lizards present, their ability to host immature *Ixodes* ticks and their ability to maintain *B. burgdorferi* infections. In the southeastern states, where the incidence of Lyme disease is much lower than in the mid-Atlantic region, immature *I. scapularis* feed primarily on lizards (Apperson et al. 1993). Laboratory experiments have demonstrated that *I. scapularis* from northern states will feed on lizards (James and Oliver 1990), indicating the potential for these ticks to feed on lizards where they coexist. Therefore, we hypothesized that immature *I. scapularis* would feed on lizards in Maryland, and have the potential to affect the enzootic cycle. This is the first report of *B. burgdorferi* s.s. detected in lizards in the mid-Atlantic region.

At least seven species of lizards are indigenous to Maryland (Behler and King 1979). We sampled three species which are found in the southern and eastern regions of the state. All three species in southern Maryland were parasitized by subadult *I. scapularis*. The ability of

lizards to maintain *B. burgdorferi* infection varies by species. Southeastern five-lined skinks (*E. inexpectatus*) and green anoles (*Anolis carolinensis*), both of which are native to the southeastern United States, can be infected with *B. burgdorferi* by needle inoculation and tick bite (Levin et al. 1996). *Borrelia burgdorferi* was recovered from these lizards using xenodiagnosis with larval *I. scapularis* which were able to subsequently infect mammals as nymphs (Levin et al. 1996). In direct contrast, Lane and Quistad (1998) found that the borreliacidal factor present in *S. occidentalis* prevented *B. burgdorferi* from surviving. In our study, *B. burgdorferi* s.s. was detected in more than a quarter (27.6%) of the lizards examined, representing all three species sampled. The genospecies identification was confirmed as *B. burgdorferi* s.s. by phylogenetic analysis. Despite extensive studies, no other genospecies have been recovered in the study region (Anderson and Norris 2006, Anderson et al. 2006, D.E. Norris unpublished data). We were unable to determine whether the *B. burgdorferi* gene fragments amplified from our field-caught lizards represented viable organisms that could subsequently infect ticks since positive samples were determined by PCR alone and not by culture. However, as observed in this study and others, ticks are most frequently attached near the shoulders and legs on lizards (Lane and Loye 1989, Oliver et al. 1993). Therefore, recovery of *B. burgdorferi* DNA from a tail snip suggests that the bacterial infection was disseminated. This is congruent with findings by Clark et al. (2005) in which *B. burgdorferi* DNA was detected in tail blood. Because dissemination of *B. burgdorferi* in lizards normally takes several weeks (Levin et al. 1996) and lizards are only infested with immature ticks, the *B. burgdorferi*-positive lizards would have to have been infected by nymphs during a previous transmission season when immature ticks were active, nearly a year prior to capture in this study. Further studies are needed to determine whether *B. burgdorferi* isolated from the three lizard species is viable, can be acquired by feeding ticks, and can infect other potential lizard or rodent reservoirs.

All ticks collected from the lizards were subadult. Despite 27.6% of the lizards being positive for *B. burgdorferi* s.s., only one positive tick, an unfed, flat nymph, was recovered. These findings suggest that either the lizards collected were not infectious, or that *Borrelia* present were below the detection threshold in these ticks. Although *B. burgdorferi* s.s. was detected in some of the lizards caught, the number of lizards examined was limited and we were not able to reach conclusions about their effects on the maintenance of *B. burgdorferi*. In order to fully determine the effect the lizards have on the enzootic cycle of *B. burgdorferi*, the infection status, tick burden, and population abundance of both *P. leucopus*, a major host for ticks and reservoir for *B. burgdorferi* in the region, and the lizards from the same collection locations need to be examined. The rate at which ticks feed on lizards and the ability of lizards to disrupt the rodent-tick transmission cycle may be strongly influenced by the relative density of lizards to preferred mammalian hosts. These host densities are currently unknown. Because reservoir competence appears to vary by species of lizard (Lane and Quistad 1998, Levin et al. 1996), the other lizard species present in Maryland should also be evaluated for the presence of ticks and *B. burgdorferi* s.s.

Despite the preliminary nature of these findings, our results suggest that reptiles have the potential to play a role in the transmission cycle of *B. burgdorferi* s.s. in endemic regions in Maryland. The results of this study confirm that nonmammalian vertebrates serve as hosts

for *Ixodes* ticks and suggest that they have the potential to serve as reservoirs for *B. burgdorferi*, resulting in a more complex enzootic cycle in this region than previously considered. The role of alternative hosts on established maintenance and transmission cycles for *B. burgdorferi* needs to be more thoroughly assessed in the mid-Atlantic region by studying the contributions of both mice and lizards.

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Table 1

Lizard Basics: Location of Capture, Species, and Presence of Ticks for Lizards

<i>Lizard number</i>	<i>Species</i>	<i>Borrelia burgdorferi positive</i>	<i>Ticks present</i>	<i>Collection site</i>	<i>County of collection</i>
2003070109	<i>Eumeces laticeps</i>	No	Yes	NGF	Queen Anne's
2003081404	<i>Eumeces fasciatus</i>	No	Yes	WF	Queen Anne's
2003062301	<i>Sceloporus undulatus</i>	Yes	No	LSP	Charles
2003043001	<i>Sceloporus undulatus</i>	No	No	LSP	Charles
2003050701	<i>Sceloporus undulatus</i>	No	Yes	LSP	Charles
2003050801	<i>Sceloporus undulatus</i>	No	Yes	LSP	Charles
2003090907	<i>Sceloporus undulatus</i>	No	No	LSP	Charles
2003090908	<i>Sceloporus undulatus</i>	No	No	LSP	Charles
2003042901	<i>Sceloporus undulatus</i>	No	No	LSP	Charles
2003043002	<i>Eumeces fasciatus</i>	No	Yes	LSP	Charles
2002071604	<i>Sceloporus undulatus</i>	No	No	LSP	Charles
2002072201	<i>Sceloporus undulatus</i>	No	No	SMWS	St. Mary's
2002052202	<i>Sceloporus undulatus</i>	Yes	No	LSP	Charles
2002071801	<i>Eumeces fasciatus</i>	No	No	PSP	Charles
2002081901	<i>Sceloporus undulatus</i>	Yes	No	SMWS	St. Mary's
2002082003	<i>Sceloporus undulatus</i>	No	No	SMWS	St. Mary's
2002071704	<i>Sceloporus undulatus</i>	No	No	LSP	Charles
2002071501	<i>Sceloporus undulatus</i>	No	No	LSP	Charles
2002052905	<i>Eumeces laticeps</i>	Yes	No	GW	St. Mary's
2002062518	<i>Eumeces fasciatus</i>	Yes	Yes	GW	St. Mary's
SMC1	<i>Eumeces fasciatus</i>	Yes	Yes	CHR	St. Mary's
SMC2	<i>Eumeces fasciatus</i>	No	Yes	CHR	St. Mary's
SMC3	<i>Eumeces fasciatus</i>	Yes	Yes	CHR	St. Mary's
SMC4	<i>Eumeces fasciatus</i>	No	No	CHR	St. Mary's
SMC5	<i>Eumeces fasciatus</i>	Yes	Yes	CHR	St. Mary's
SMC6	<i>Eumeces fasciatus</i>	No	No	BCR	St. Mary's
SMC7	<i>Sceloporus undulatus</i>	No	No	H235	St. Mary's
SMC8	<i>Eumeces fasciatus</i>	No	No	IBR	St. Mary's
SMC9	<i>Eumeces fasciatus</i>	No	Yes	LBR	St. Mary's

Table 2

Primers Used and Source

Primer name	Sequence	Source	Expected product size
Lizard 12S forward	5'-AGAGGAGCCTGTCCTATAATCG-3'	this study	298 bp
Lizard 12S reverse	5'-GTGACGGGGGTGTGT-3'	this study	
<i>flaB</i> exterior forward	5'-AAGTAGAAAAAGTCTTAGTAAGAAATGAAGGA-3'	Johnson et al. 1992	611 bp
<i>flaB</i> exterior reverse	5'-AAGCATACTAAGTACTATTCTTTATAGA-3'	Johnson et al. 1992	
<i>flaB</i> interior forward ^a	5'-CACATATCAGATGCAGACAGAGGTTCTA-3'	Johnson et al. 1992	390 bp
<i>flaB</i> interior reverse ^a	5'-GAAGGTGCTGTAGCAGGTGCTGGCTGT-3'	Johnson et al. 1992	
<i>ospA</i> exterior forward	5'-AAAAAATATTTATTGGGAATAGG-3'	Guttman et al. 1996	702 bp
<i>ospA</i> exterior reverse	5'-GTTTTTTTGTGCTGTACACTAAATGTATAA-3'	Guttman et al. 1996	
<i>ospA</i> interior reverse ^a	5'-GCTTAAAGTAACAGTTCC-3'	Guttman et al. 1996	345 bp
<i>ospA</i> interior forward ^a	5'-GG AGTACTTGAAGGCG-3'	Guttman et al. 1996	
<i>ospC</i> exterior forward	5'-AAAGAATACATTAAGTGCCATATT-3'	Wang et al. 1999	597 bp
<i>ospC</i> exterior reverse ^a	5'-GGGCTTGTAAAGCTCTTTAACTG-3'	Wang et al. 1999	
<i>ospC</i> interior forward ^a	5'-TTGTTAGCAGGAGCTTATGCAATATC-3'	Wang et al. 1999	314 bp
<i>rrf-rrl 23SN1</i>	5'-ACCATAGACTCTTATTACTTTTGAC-3'	Rijpkema et al. 1995	380 bp
<i>rrf-rrl 23SC1</i>	5'-TAAGCTGACTAATACTAATTACCC-3'	Rijpkema et al. 1995	
<i>rrf-rrl 5SCB</i> ^a	5'-GAGAGTAGGTTATTGCCAGGG-3'	Rijpkema et al. 1995	225 bp
<i>rrf-rrl 23SN2</i> ^a	5'-ACCATAGACTCTTATTACTTTGACCA-3'	Rijpkema et al. 1995	

^aDenotes primers used in DNA sequencing.

Table 3

PCR Results of Lizard Samples

Lizard ID	12s	flaB	ospA	ospC	rrf-rrl
2003070109	+	-	-	-	-
2003081404	+	-	-	-	-
2003062301	+	-	-	+	-
2003043001	+	-	-	-	-
2003050701	+	-	-	-	-
2003050801	+	-	-	-	-
2003090907	+	-	-	-	-
2003090908	+	-	-	-	-
2003042901	+	-	-	-	-
2003043002	+	-	-	-	-
2002071604	+	-	-	-	-
2002072201	+	-	-	-	-
2002052202	+	-	-	-	+
2002071801	+	-	-	-	-
2002081901	+	-	-	+	+
2002082003	+	-	-	-	-
2002071704	+	-	-	-	-
2002071501	+	-	-	-	-
2002052905	+	-	-	+	-
2002062518	+	-	-	+	-
SMC1	+	+	-	-	-
SMC2	+	-	-	-	-
SMC3	+	-	+	-	-
SMC4	+	-	-	-	-
SMC5	+	-	+	-	+
SMC6	+	-	-	-	-
SMC7	+	-	-	-	-
SMC8	+	-	-	-	-
SMC9	+	-	-	-	-

PCR, polymerase chain reaction.