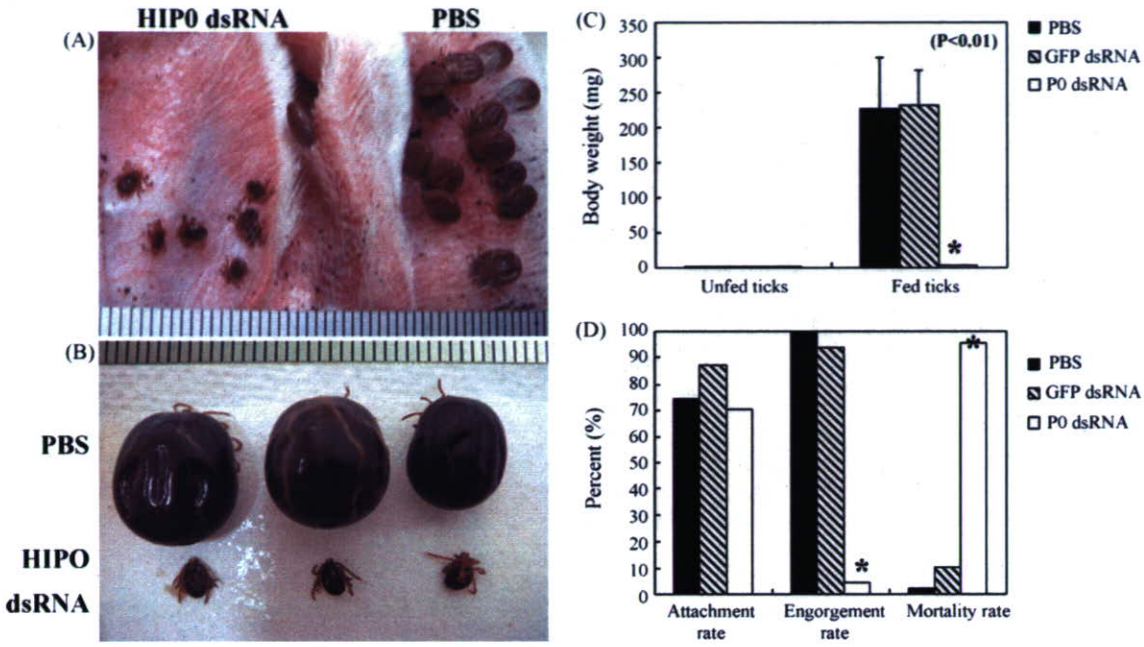

Fig. 6. Effect of HlP0 RNAi on feeding and survival of adult ticks. Comparison of the size of 4-day-fed (A) and detached ticks (B) between the control and HlP0 dsRNA-treated groups, showing that both partially fed ticks and finally detached ticks in the HlP0 dsRNA-treated group were much smaller than those in the control group. (C) Comparison of the fed body weight of unfed and detached ticks in each group. The fed body weight of HlP0 dsRNA-injected ticks was remarkably lower (shown with a *star*) than that of ticks in the control groups. (D) Comparison of the attachment rate 2 days post-infestation, engorgement rate, and mortality in each group. HlP0 dsRNA-treated ticks showed lower engorgement rate (4%) and higher mortality (96%) (shown *with stars*) than PBS- and GFP dsRNA-treated ticks.

After feeding, 2.5% of PBS- and 10.4% of GFP dsRNA-treated ticks died; in contrast, 96% of HlP0 dsRNA-treated ticks died (Fig. 6D). PBS- and GFP dsRNA-injected ticks started to lay eggs 5 days post-engorgement, while no data could be obtained from HlP0 dsRNA-treated ticks because of the death of the ticks.

In order to investigate the impact of RNAi on the morphology of tissues from ticks, salivary glands, and cuticle were dissected and compared under the microscope. As shown in Fig. 7A, salivary glands from HlP0 dsRNA-injected ticks obviously exhibited a smaller size of acini than those of PBS-injected ticks. Moreover, the cuticle of HlP0 dsRNA-injected ticks was thinner and less symmetrical than that of the control group (Fig. 7B).

4. Discussion

In the present study, a full-length of the 1141 bp ribosomal protein (HlP0) gene was isolated from the tick *H. longicornis*. This is the first report to date of P0 from a tick or from any other arachnid. The gene encodes a putative 320 amino acid residue with a molecular mass of 35 kDa. The predicted HlP0 protein was supposed to contain three domains: the rRNA-binding domain, the P1/P2 protein-binding domain, and a highly conserved C-terminal region. It was reported that vaccination with a peptide of C-terminal domain from *Plasmodium falciparum* protected mice against a malaria parasite challenge (Rajeshwari et al., 2004). In a similar way, it is possible that the inoculation of hosts with the C-terminal domain of HlP0 from *H. longicornis* may also provide some protection against tick infestation.

In this study, rHlP0/Trx rather than rHlP0/GST was used to produce antibodies for the detection of native HlP0 protein in different tissues from ticks. This decision was based on the consideration that anti-rHlP0 sera developed from the rHlP0/GST protein can recognize the GST protein in the tissues, since GST is ubiquitously expressed in ticks (da Silva Vaz Jnr et al., 2004). The results of both Western blotting and RT-PCR indicated that the HlP0 gene transcript was expressed in all tested tissues.

In a previous study, it was indicated that P1/P2 could be knocked out in yeast and the cells were still viable with a remaining copy of P0, while removal of P0 was lethal (Santos and Ballesta, 1994). This lethality of the disruption of HlP0 mRNA was also demonstrated in

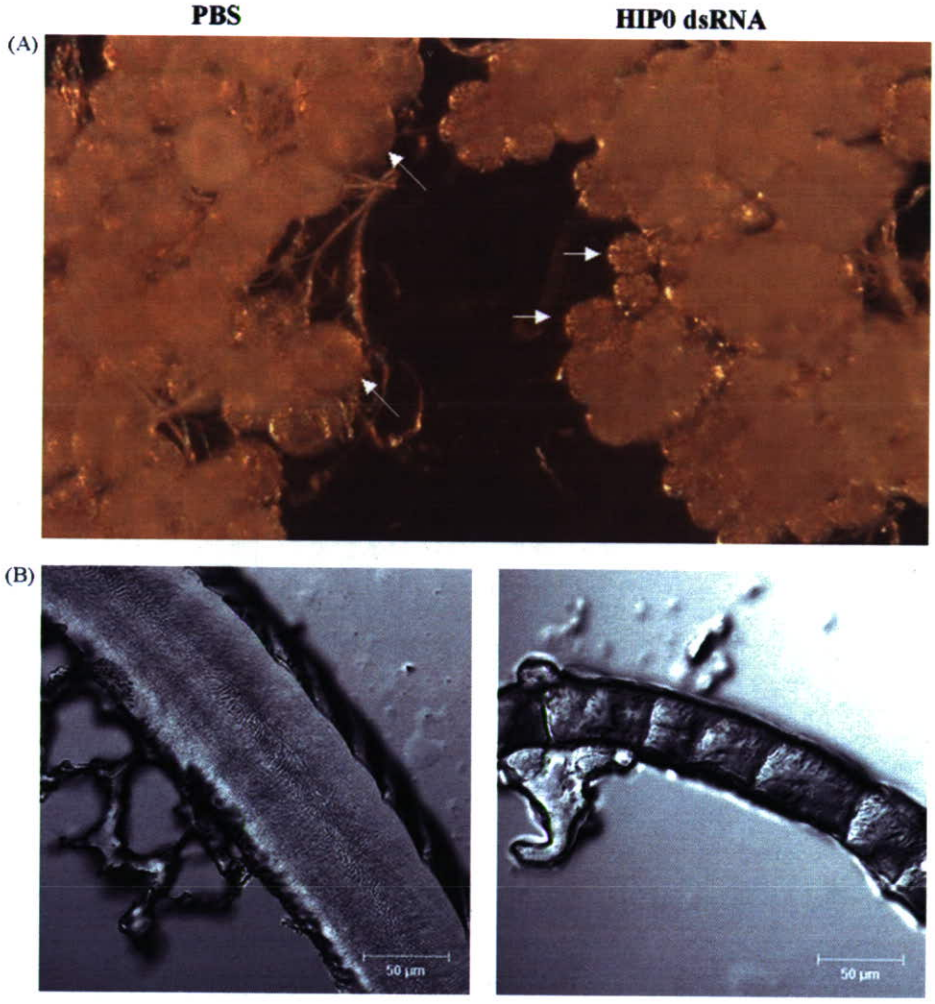

Fig. 7. Morphological effect of HlP0 RNAi on the salivary glands and cuticle of ticks. (A) Difference of the salivary glands between PBS- and HlP0 dsRNA-treated ticks. The granular salivary gland acini are shown with *arrows*. (B) Difference of the cuticle between PBS- and HlP0 dsRNA-treated ticks.

ticks by dsRNA treatment in this study. RNAi of the HlP0 gene blocked the feeding of ticks and led to the death of ticks; even the silencing of the HlP0 gene was incomplete. Incomplete silencing of the gene seems to take place frequently in dsRNA interference experiments (Gotta and Ahringer, 2001). In order to exclude the possibility of a toxic effect of dsRNA, GFP dsRNA was employed as a negative control (Ghanim et al., 2007), and similar body size of partially fed and engorged ticks as well as similar size of salivary glands and cuticle to those of PBS-treated ticks was observed (data not shown). Interestingly, GFP dsRNA-treated ticks indicated a little faster migration of actin protein and lower relative expression of HlP0 protein than those from PBS-treated group (as shown in Fig. 5) because of unknown reason, which deserve further investigation. Moreover, 10 HlP0 dsRNA-injected ticks were kept at 25 °C in a moist chamber for more than 12 days, and none died (data not shown). The death of fed ticks in the HlP0-silenced group was probably due to the disability of a large subunit in cell growth or apoptosis. After P0 repression in yeast, a dramatic reduction in the amount of polysomes was observed in the cell extracts, and half-mers in the polysome population appeared, which indicated a deficit in the amount of active large subunits available (Santos and Ballesta, 1994). As described, the cleavage of 28S RNA is thought to be an important step in the process of apoptosis, and there is a positive, though incomplete, correlation between rRNA cleavage and internucleosomal DNA fragmentation (Houge and Doskeland, 1996). Since P0 directly binds 28S RNA, it is possible that repression of HlP0 resulted in the same effect as rRNA cleavage. Furthermore, the P0 protein has both 3′ and 5′ APE activity, acts on abasic DNA, and

shows strong DNase activity for both single- and double-stranded DNA (Yacoub et al., 1996; Frolov and Birchler, 1998), which is important for repair of damaged DNA. The knockdown of HlP0 gene may thus lead to the accumulation of damaged DNA. Primary observations in mosquito cells indicated that silencing of P0 resulted in the fragmentation of DNA, a sign of apoptosis (Jayachandran and Fallon, 2003).

After feeding, the HlP0 dsRNA-injected ticks achieved a conspicuously lower body weight (1.32-fold vs. 113.38-fold and 116.16-fold, calculated by the fed body weight/unfed body weight) and a remarkably smaller size of salivary gland acini and cuticles than that of the two control groups. This suggests that the function of the salivary gland was impacted and synthesis of the new cuticle was obviously blocked by the knockdown of HlP0 gene. Salivary gland is an important organ for the feeding of ticks, where various bioactive molecules are synthesized and secreted into saliva during feeding, i.e., anti-platelet factors, anticoagulant proteins, and anti-inflammatory proteins (Valenzuela, 2004). In cells from acinus II of unfed ticks, many free ribosomes were observed, and cisternae of rough endoplasmic reticulum (RER) were not distended, while, in 2-day-fed ticks, the cells became larger and contained dilated cisternae of RER (Sonenshine, 1991). We can suspect that binding of 40S and 60S particles occurred during this period, which could be attributed to the 28S RNA-binding activity of P0. However, this binding was supposed to be impeded by the disruption of HlP0 mRNA, which led to the weak synthesis of bioactive molecules and the consequent failure of tick feeding. In previous study, the tick cuticle was demonstrated to increase in thickness during the first few days after attachment for the accommodation of a large amount of blood meal during the later phase of rapid engorgement. In phase of rapid repletion, synthesis of the new cuticle slows down or ceases (Obenchain and Galun, 1982; Sonenshine, 1991). However, in the present study, the silencing of HlP0 gene was supposed to impede the rapid cuticle synthesis in the first phase. Furthermore, it is possible that HlP0 expression in other tissues may be impaired as well. In any case, it will be interesting to continue this investigation for a further understanding of the effects in ticks induced by RNAi of HlP0 gene.

As a conclusion, a full-length gene termed HlP0 has been isolated from the tick *H. longicornis*. The deduced amino acid sequence of the HlP0 gene contains three presumptive domains. Transcripts of HlP0 exist in all developmental stages of ticks and different tissues from partially fed adult ticks. The RNAi of HlP0 gene led to the failure of blood-sucking and tick death.

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NOTE *Parasitology*

Molecular Survey of *Babesia canis* in Dogs in Nigeria

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ABSTRACT. An epidemiological study of *Babesia canis* in dogs in Nigeria was performed. Four hundred blood samples collected from dogs in Nigeria were investigated using nested PCR and sequence analysis. On nested PCR screening, nine samples (2.3%) produced a band corresponding to a 698-bp fragment indicative of *B. canis* infection. Sequence analysis of the PCR products identified eight samples (2.0%) as *B. canis rossi* and the ninth (0.3%) as *B. canis vogeli*. This is the first report of the prevalence of *B. canis rossi* and *B. canis vogeli* in dogs in Nigeria.

KEY WORDS: *Babesia canis*, Nigeria, PCR.

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Babesia canis is a tick-transmitted hemoprotozoan parasite that induces anemia, fever, jaundice, hemoglobinuria, and sometimes fatal symptoms in dogs. *B. canis* is classified into three subspecies: *B. canis rossi*, *B. canis vogeli*, and *B. canis canis* [5, 10, 11]. The arthropod vectors of *B. canis rossi*, *B. canis vogeli*, and *B. canis canis* are *Haemaphysalis leachi*, *Rhipicephalus sanguineus*, and *Dermacentor reticulatus*, respectively [5, 10, 11].

A diagnosis of canine babesiosis is made by the detection of parasites in a peripheral blood smear under a microscope or by serological tests such as an indirect fluorescent antibody test or enzyme-linked immunosorbent assay. Compared with these methods, molecular analysis using PCR has greater sensitivity and specificity in the detection of pathogens [1, 4, 8, 9, 11]. Thus, the PCR method makes it possible to diagnose babesiosis in the blood samples of dogs that show very low parasitemia, as in the early stages of infection, or in dogs with no symptoms. Moreover, it is possible to differentiate species or subspecies of pathogens by sequence analysis following PCR [2, 9–11].

Throughout the African continent, epidemiological studies of canine babesiosis using molecular methods have been reported only in South Africa and the Sudan, and *B. canis rossi* and *B. canis vogeli* have been shown to be distributed in both countries [10, 11]. *B. canis vogeli* is thought to be distributed in large parts of the tropical and subtropical regions of all continents in which its vector ticks exist [12]. Therefore, it is anticipated that this parasite is distributed widely throughout the African continent. However, the prevalence of *B. canis* infections in dogs in other parts of Africa is unknown. To determine the incidence and identify subspecies of *B. canis* in dogs in Nigeria, located in the midwest of Africa, we performed an epidemiological study using PCR and sequence analysis.

Peripheral blood samples from 400 dogs were obtained randomly from many parts of Nigeria between October 2004 and August 2005 (Table 1). Each 200 μl of blood was fixed on an FTA Card (Whatman, UK) and stored at room temperature in the dark. DNA was extracted from the blood samples on the FTA Cards using the Whatman FTA Card DNA Isolation Kit (Whatman).

Primers for the first PCR and nested PCR were designed based on a fragment of the gene that encodes *B. canis* 18S rDNA (GenBank accession nos. AY072925, AY072926, and L19079). The forward primer B18S-F (5'-TGGTTGATCCTGCCAGTA-3') and the reverse primer B18S-R (5'-CTTCTCCTTCCTTTAAGTGA-3') were used in the first amplification reaction [7]. Primers B18S-1 (5'-GGGAGGTAGTGACAAGAAA-3') and B18S-2 (5'-TTCCCCGTGTTGAGTCAAA-3') were used in the nested PCR.

The DNA extracted from the blood samples on the FTA Cards was added to a reaction mixture containing 10 pmol of each primer B18S-F and B18S-R, 5 U/μl AmpliTaq Gold

Table 1. Sex and age of dogs examined

		No. of examined
	Total	400
Sex	Male	169
	Female	227
	ND*	4
Age (yr)	0–3	336
	4–7	46
	≥ 8	14
	ND*	4

* ND: not data.

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DNA polymerase (Applied Biosystems, U.S.A.), 2.0 mM dNTPs (Applied Biosystems), 10 × PCR Gold Buffer (150 mM Tris-HCl [pH 8.0], 500 mM KCl; Applied Biosystems), and 25 mM $MgCl_2$ (Applied Biosystems). The reaction mixture was adjusted to 25 μl with ultrapure distilled water. The amplification conditions were as follows: 95°C for 10 min; 30 cycles of denaturation at 94°C for 30 s, primer annealing at 55°C for 2 min, and amplification at 72°C for 2 min; followed by a final extension at 72°C for 5 min. The nested PCR was performed using primers B18S-1 and B18S-2 under the same conditions as the first PCR. The PCR products were separated by electrophoresis on 1.5% TBE agarose gel and stained with ethidium bromide. The expected size of PCR product for *B. canis* 18S rDNA is a 698 bp fragment after nested PCR.

The amplified DNA was purified with the QIAquick® PCR Purification Kit (Qiagen, Germany), and the sequences were determined directly using the B18S-1 or B18S-2 primer with the ABI PRISM® 310 Genetic Analyzer (Applied Biosystems). The sequences obtained were analyzed with Genetyx® version 8 (Genetyx, Japan).

In the first PCR reaction using the primers B18S-F and B18S-R, no positive bands have been detected in all samples. In the nested PCR using the primer set designed in this study, nine samples (2.3%) in 400 samples showed a band of 698 bp fragment which is expected size for *B. canis* 18S rDNA. The sequences of these nine PCR products were determined (*B. canis rossi*, AB303071-AB303075; *B. canis vogeli*, AB303076). Eight samples (2.0%) showed high sequence similarity to the 18S rRNA gene of the *B. canis rossi* isolate from dog 44 (DQ111760) from the Sudan at both the nucleotide (99.1%–99.8%) and predicted amino acid levels (98.7%–99.1%). Another sample (0.3%) was similar in its sequence to a *B. canis vogeli* 18S rRNA gene from Italy (AY072925) at both the nucleotide (99.6%) and predicted amino acid levels (98.7%). Moreover, this sequence also showed partial sequence homology to a *B. canis vogeli* 18S rRNA gene isolated in South Africa (AF547387) at both the nucleotide (99.6%) and predicted amino acid levels (98.7%), and to the sequence of a *B. canis vogeli* 18S rRNA gene from the Sudan (DQ111765) at both the nucleotide (99.6%) and predicted amino acid levels (98.9%).

In this study, eight of 400 dogs (2.0%) were positive for *B. canis rossi* and one dog (0.3%) was positive for *B. canis vogeli*. In contrast, the incidence of *B. canis* in dogs in South Africa and the Sudan were 14.8% and 9.0%, respectively [10, 11]. Thus, the infection rates of *B. canis rossi* and *B. canis vogeli* are lower in Nigeria than those in South Africa or the Sudan. *B. canis rossi* and *B. canis vogeli* are known to be transmitted by ticks, and the rates of tick infestations in dogs in South Africa and the Sudan are reported to be 72.2% and 78.2%, respectively [6, 11]. The arthropod vectors of *Babesia* spp., such as *H. leachi* and *R. sanguineus*, have been confirmed to occur widely in South Africa and the Sudan [6, 10, 11]. A lower rate of tick infestation in dogs in Nigeria might contribute to their lower rates of infection with *B. canis rossi* and *B. canis vogeli*. However, there has been no report of the occurrence of the tick vectors of *Babesia* spp., such as *H. leachi, R. sanguineus*, and *D. reticulatus*, in Nigeria. The rate of dogs infested with ticks should be investigated, and the transmission of *B. canis rossi* and *B. canis vogeli* by these ticks in Nigeria confirmed.

Table 2. Dogs positive for *Babesia canis* in Nigeria

Dog no.	Breed	Sex	Age (yr)	Subspecies
2	Alsatian	Male	0.2	rossi
8	Rottweiler	Female	0.3	rossi
23	Local	Female	1	rossi
25	Doberman	Male	3	rossi
127	Local	Male	2	rossi
135	Local	Female	1	rossi
266	Alsatian	Male	2	vogeli
354	Local	Female	3	rossi
398	Alsatian	Male	0.4	rossi

In this study, young dogs under three years were positive for *B. canis* (Table 2). Moreover, it is noteworthy that three samples from puppies under six months were also positive (Table 2; dogs 2, 8, and 398). Fukumoto *et al.* [3] have demonstrated transplacental infection of *B. gibsoni* in dogs. The *B. canis* infections of young dogs recorded in this study suggest that this parasite is also vertically transmitted from dogs to their puppies. However, there are few samples from old dogs, and more samples from dogs over four years should be examined.

In conclusion, the primers designed in this study are useful for the detection of *B. canis*. PCR with these primers and sequence analysis have demonstrated that *B. canis rossi* and *B. canis vogeli* exist in dogs in Nigeria.

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