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Absence of symbiotic *Wolbachia* endobacteria in *Setaria cervi* from Birbhum, West Bengal, India

Wolbachia are symbiotic endobacteria that exist in the lateral cords of adult females as well as microfilariae of most filarial nematodes, including *Dirofilaria immitis*, *Litomosoides sigmodontis*, *Onchocerca volvulus*, *Wuchereria bancrofti* and *Brugia malayi*[1,2]. *Wolbachia* are causative agents for a variety of modifications in host development and reproduction, besides being implicated to contribute to disease progression. In recent years, studies have linked tetracycline treatment of filarial-infected animals with reduced worm burdens and decreased level of microfilaremia. This has been demonstrated in an animal model with *Litomosoides sigmodontis* and recently confirmed in patients infected with *O. volvulus*[3,4]. Depletion of the endosymbionts drastically affects worm fertility and has led to the definition of *Wolbachia* as a target for a novel chemotherapeutic approach for human onchocerciasis with doxycycline[4].

Singhal and co-authors[5–7] tested different antifilarial agents on *Setaria cervi* in rats and reported that it is a suitable model for screening antifilarial agents. According to Singhal *et al.*[7], this is a suitable model because the rate of proper establishment of transplanted parasites in rats is 50% and microfilaria appear in the peripheral blood within 10–15 days after transplantation and persist for 120 days. We have reported filaricidal properties of acaciaside A and acaciaside B, the two triterpenoid saponins isolated from the funicles of *Acacia auriculiformis* using this model. Since the *S. cervi* rat model has been used for screening antifilarial agents, it is of great interest to know whether symbiotic *Wolbachia* endobacteria are present in adult worms. Here we report absence of symbiotic *Wolbachia* in *S. cervi* collected from Birbhum, West Bengal, India.

Adult worms of *S. cervi* were collected from the peritoneal cavity of freshly slaughtered cows at local abattoirs (Kashipur, Birbhum), washed briefly with modified Ringers medium at 37°C and stored immediately in 1 ml of TEN buffer (100 mM Tris, 5 mM EDTA, 200 mM sodium chloride; pH 7.5) at –20°C. Total genomic DNA was extracted from adult parasites following the method by Smith and Rajan[8], with slight modifications. A small piece of frozen worm (5 mg of tissue) was thawed and resuspended in 500 μl of lysis buffer, pH 8.0 (20 mM Tris-HCl, 50 mM EDTA, 0.5% SDS; 100 mM NaCl, 1%(v/v) β-mercaptoethanol) containing proteinase-K 0.1 mg/ml and incubated at 55°C for 3 h. To inactivate proteinase-K, samples were heated at 95°C for 10 min. After phenol-chloroform-isoamyl alcohol extraction and ethanol precipitation, the pellet was washed with cold ethanol (70%) and then resuspended in sterile 25 μl 10 mM TE buffer (pH 8.0). Total genomic DNA was also extracted from *D. immitis* microfilariae, collected from blood drawn from naturally infected stray dogs, as described above.

Polymerase chain reaction (PCR) was performed to check DNA integrity and to assay for the presence of *Wolbachia* organisms in *S. cervi*. PCR was performed in 50 μl of reaction mixture containing 1X PCR buffer with $(NH_4)_2SO_4$ (Fermentas), 2 mM dNTP mix, 2 mM $MgCl_2$, 20 ng/ml each of forward and reverse primers and 1.5 units *Taq* polymerase (Fermentas). PCR products were visualized by running 5 μl of reaction mixture in 1% agarose gel followed by staining with ethidium bromide. To confirm filarial DNA[8], primers were derived from 28S rRNA. The amplified product was 150 bp using an annealing temperature 50°C. For confirmation of eubacterial DNA integrity[8], primers were derived from 16S rRNA. The amplified product was 1.4 kb and the annealing temperature was 59°C. *Wolbachia* DNA was confirmed using *Wolbachia* 16S rRNA primer[8] with an annealing temperature 51°C and the amplified product was 207 bp. *D. immitis* adult and microfilaria are known to harbour *Wolbachia* endosymbiont[2]. Thus DNA extracted from *D. immitis* microfilariae was used as positive control in the present study and sterile distilled water instead of template DNA was used as negative control.

In the present study using filarial primers, PCR amplification of *S. cervi* and *D. immitis* genomic DNA yielded distinctive bands at 150 bp corresponding to filarial 28S rRNA gene product (lane 3, Figure 1 and lane 2, Figure 2), establishing the quality of the template DNA and authenticity of the experimental protocol. However, use of 16S rDNA primer specific for *Wolbachia* did not produce any amplified product (band) when *S. cervi* genomic DNA preparation was used as template even after 35 cycles of amplification (lane 5, Figure 1). In a similar condition, *D. immitis* genomic DNA used as template gave a distinct 16S rDNA or

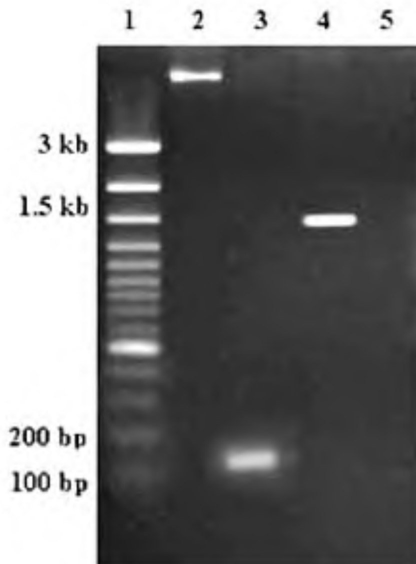

Figure 1. Polymerase chain reaction of *Setaria cervi* total genomic DNA to assay for the presence of *Wolbachia* endosymbiont. PCR amplification using primers specific for filarial 28S rRNA (lane 3) and eubacterial 16S rRNA (lane 4) sequences yielded products of 150 bp and 1.4 kb respectively. No band corresponding to *Wolbachia*-specific 207 bp found through PCR amplification using *Wolbachia* 16S rRNA primer in *S. cervi* (lane 5). Electrophoretic separation of *S. cervi* genomic DNA (lane 2) as well as migration pattern of DNA ladder (lane 1, Fermentas) is also shown.

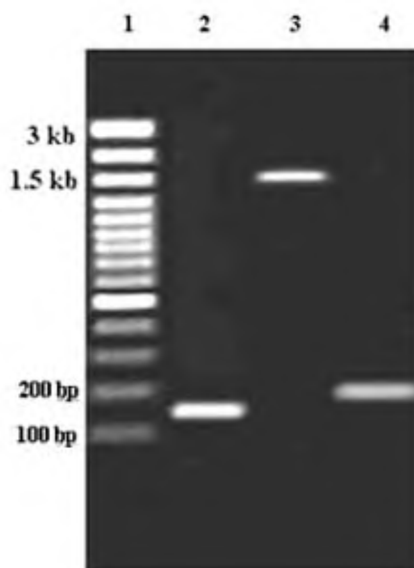

Figure 2. Polymerase chain reaction of *Dirofilaria immitis* total genomic DNA using primers specific for *Wolbachia* 16S rRNA (lane 4), filarial 28S rRNA (lane 2) and eubacterial 16S rRNA (lane 3). Presence of a distinct band of size approximately 207 bp confirms the presence of *Wolbachia* in microfilariae of *D. immitis*. Migration pattern of DNA ladder (lane 1, Fermentas) is shown at extreme left.

Wolbachia-specific amplified product in agarose gel (lane 4, Figure 2). These results suggest that *Wolbachia* sp. are either absent from *S. cervi* or present in extremely low numbers.

Analysis of filarial parasites and their endosymbiont, i.e. antialpha proteobacteria *Wolbachia* sp., indicates a long-term and stable association between the two organisms. This obligatory symbiotic relationship is evident from several reports on filarial worms, where elimination of *Wolbachia* by treatment with tetracycline and other antibiotics decreases host fitness in several nonspecific ways, including survival, developmental success and reproduction[3]. The results of *Acanthocheilonema viteae*, a *Wolbachia*-free filarial worm, contrast with those observed on filarial worms containing *Wolbachia*[4,9], suggesting that the effect of the antibiotics is related to the elimination of the bacteria and not simply to the antibiotics. Although the above may explain the view that *Wolbachia* sp. are essential symbionts of filarial nematodes, we have now reported a fifth filarial nematode found to be free of *Wolbachia* sp. Only four nematodes have previously been proven negative for *Wolbachia* sp. by PCR, electron microscopy or immunohistology. These are *A. viteae*[10], *O. flexuosa*[11], *Loa loa*[12] and *S. equina*[13]. It is therefore likely that *Setaria* species have always been free of *Wolbachia* sp. Lack of *Wolbachia* in all five filarial nematodes studied so far, suggests that the symbiotic relationship is not necessary for the successful development and reproduction of all filarial parasites.

We have shown[14] that the mixture of acaciaside A and acaciaside B, the two triterpenoid saponins isolated from the funicles of *A. auriculiformis*, killed *in vitro* and *in vivo* microfilariae and adults of *S. cervi*. Since this cattle parasite does not contain *Wolbachia*, it is likely that the filaricidal activity of saponins may be mediated through a different target altogether. These two saponins are known to interact with the membrane, thus inflicting membrane damage[15]. Our findings on the mechanism of action of saponins further revealed that superoxide anion is probably involved in the expression of membrane-damaging effect of the saponins[16].

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