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Infection of the intermediate mite host with *Wolbachia*-depleted *Litomosoides sigmodontis* microfilariae: Impaired L1 to L3 development and subsequent sex-ratio distortion in adult worms

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Abstract

The rodent filaria *Litomosoides sigmodontis* harbour *Wolbachia*, endosymbionts essential for worm embryogenesis, larval development and adult survival. To study the effect of tetracycline, which depletes *Wolbachia*, on the development of microfilariae (L1s, MF) to L3 in the intermediate host *Ornithonyssus bacoti*, and to observe the development of *Wolbachia*-depleted L3s in Mongolian gerbils (*Meriones unguiculatus*); microfilaremic gerbils were treated orally with tetracycline for 6 weeks (primary infected (1°) Tet) or untreated (1° Con). Treatment resulted in a significant reduction of *Wolbachia* per MF in 1° Tet gerbils. Naïve mites then fed on the 1° Tet and 1° Con gerbils in the week after treatment ended, when MF levels were not significantly different, and used to infect new gerbils (secondary infected (2°) Tet, 2° Con) via natural infection. The infection rate from dissected mites was 9% and 54% (1° Tet and 1° Con, respectively). After 3 months, worms were isolated from 2° gerbils. Significantly fewer female worms developed in 2° Tet gerbils. In contrast, there was no difference in the number of male worms that developed in 2° gerbils, resulting in a male biased sex-ratio. Although 2° Tet male worms had fewer *Wolbachia* than 2° Con males, development was not impaired. Female worms that developed from *Wolbachia*-depleted MF had *Wolbachia* levels equivalent to worms from 2° Con animals. Thus, tetracycline pre-treatment selected for female worms with high numbers of *Wolbachia*, whereas male worms had median *Wolbachia* levels significantly lower than 2° Con males. Therefore, female worms require a higher threshold of *Wolbachia* for their development. The worms analysed were only exposed to tetracycline as MF, ruling out direct effects of tetracycline during larval development in the mites or 2° gerbils, suggesting that the depletion of *Wolbachia* in MF was the cause of impaired larval development.

Keywords: *Litomosoides sigmodontis*; *Wolbachia*; Tetracycline; Real-time PCR; *FtsZ*

1. Introduction

Filarial infections cause a wide range of clinical symptoms including lymphoedema, hydrocele, elephantiasis, dermatitis and blindness. Lymphatic filariasis (LF) affects more than 120 million people (WHO, 2006) and onchocerciasis affects as many as 37–40 million people (WHO, 2007) with 1.3 billion people in tropical countries at risk for either disease. *Wolbachia* endosymbionts of filarial nematodes have gained great interest among filarial researchers because of their significant role in the development and reproduction of filarial nematodes (Bandi et al., 1999; Hoerauf et al., 1999, 2000; Casiraghi et al., 2001). As well as arthropods, *Wolbachia* have been found in species of the family Onchocercidae, including the major human filarial nematodes *Onchocerca volvulus*, *Wuchereria bancrofti* and *Brugia malayi* (Taylor et al., 1999; Casiraghi et al., 2005). Recently, administration of doxycycline to *O. volvulus* and *W. bancrofti*-infected patients has shown promising results of higher macrofilaricidal and embryo toxic activity to these filarial worms and ameliorates some of the pathology in

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lymphatic filariasis (Hoerauf et al., 2000, 2001, 2003; Taylor et al., 2005a,b; Debrah et al., 2006a,b). The exact mechanism underlying the endosymbiosis between *Wolbachia* bacteria and their host remains unclear.

Tetracycline treatment leads to depletion (>85%) of *Wolbachia* which results in filarial growth retardation if the treatment is started at the onset of infection, and infertility if the treatment is started after adult worms have developed (Hoerauf et al., 1999; Volkmann et al., 2003). Much work has been done showing the requirement of *Wolbachia* for oogenesis, embryogenesis and adult worm survival (Hoerauf et al., 1999, 2000, 2001, 2003). Earlier studies on the antifilarial effect of tetracycline showed that depletion of *Wolbachia* by tetracycline affects development of larval stages in the mammalian host. Tetracycline administered concomitant with *Litomosoides sigmodontis* infection lead to significant growth retardation (Hoerauf et al., 1999). Similarly, oral tetracycline treatment inhibited *Brugia pahangi* development from L3s to adult worms and this effect occurred during early larval development suggesting that tetracycline treatment interferes with the molting of larvae (Bosshardt et al., 1993; Chirgwin et al., 2003). Casiraghi et al. (2002) showed that tetracycline treatment targeting the different time points of male and female worm development (i.e., treatment before/after L4 molting) resulted in a sex-ratio distortion, suggesting that *Wolbachia* may play a more active role in female than in male worms.

However, the results described above may have arisen from a direct effect of tetracycline on the larvae rather than from the depletion of *Wolbachia* causing reduced larval development. This is supported by the shorter treatment times compared with the 3–4 weeks needed to sterilize adult worms (Hoerauf et al., 2003; Debrah et al., 2006a,b; Turner et al., 2006). Additionally, a modified tetracycline with no antimicrobial activity inhibited the L3–L4 molt, suggesting possible direct pharmacological action of tetracycline on the L3 independent of its anti-wolbachial activity (Smith and Rajan, 2000). To differentiate between a direct effect of tetracycline and the absence of *Wolbachia* on larval molting and adult worm development, we depleted *Wolbachia* from MF, knowing that tetracycline has no effect on MF viability in blood (Chirgwin et al., 2003; Debrah et al., 2006a,b). Gerbils were then infected with mites carrying infective L3s that developed from *Wolbachia*-depleted MF to study the outcome of male and female worm development. Our results show a different requirement of *Wolbachia* in the development of male and female worms with *Wolbachia* being more essential for the development of female worms than male worms.

2. Materials and methods

2.1. Animal infections and tetracycline treatment

Ethical clearance for animal experiments was approved by the regional authority in Cologne, Germany (AZ 50.203.2-BN15,40/04). Mongolian gerbils (*Meriones unguiculatus*) were maintained at the Institute for Medical Microbiology, Immunology and Parasitology, University Clinic Bonn, Germany following established University Clinic Bonn guidelines. Gerbils (8–12 weeks old) were infected with *L. sigmodontis* by mites (*Ornithonyssus bacoti*) carrying infective L3s (Hoerauf et al., 1999). Tetracycline treatment was started 3 months p.i. when MF were detectable in the blood. Before tetracycline treatment, the MF count was determined as described previously (Hoerauf et al., 1999). The microfilaraemic blood was then used to extract MF DNA for *Wolbachia* real-time, quantitative PCR (qPCR) to assess the initial *Wolbachia* load per MF. All the treated animal groups, designated as "1° Tet", received tetracycline dihydrochloride (tetracycline–HCl, Sigma–Aldrich, Taufkirchen, Germany) orally at 0.5% (w/v) in drinking water for 6 weeks. The medicated drinking water was prepared fresh daily. Controls, designated as "1° Con", were infected but left untreated. In each primary infection experiment, both 1° Tet and 1° Con groups consisted of five gerbils. At the end of treatment, blood was collected from gerbils for monitoring microfilaraemia as described above. DNA was then extracted from blood-borne MF for qPCR.

One and 3 months post-tetracycline treatment, blood was collected from 1° Con and 1° Tet gerbils to monitor microfilaraemia and *Wolbachia* levels to evaluate tetracycline treatment. After confirming a significant reduction in *Wolbachia* content per MF (>85% compared with controls) by tetracycline treatment, the 1° Tet and 1° Con gerbils were used to infect naïve mites (*O. bacoti*) as described previously (Hoerauf et al., 1999). Populations of mites that fed on 1° Tet gerbils and 1° Con gerbils were designated as "Tet" or "Con" mites, respectively. After 14 days, 100 mites from Tet and Con mites were dissected and examined for the presence of L3s to monitor success in molting from L1 to L3. Additionally, 50 L3s isolated from Tet and Con mites were pooled in batches of 10. DNA was extracted from the pooled L3s and used for quantification of *Wolbachia* using qPCR. The remaining Tet and Con mites (approximately 200 with one or one to four larvae, respectively) were used to infect naïve gerbils (2° Tet, five to seven gerbils; 2° Con, five to seven gerbils) designated as secondary infections. These 2° Tet and 2° Con gerbils were reared for 3 months to study the outcome of adult worm development. Before necropsy, blood was collected from Tet and Con gerbils to monitor microfilaraemia and *Wolbachia* levels in MF.

2.2. Worm recovery at necropsy

Three months after the secondary infection, the gerbils were sacrificed and the worms removed from the pleural cavity. Worms were washed twice in sterile PBS to remove blood cells and other debris. Worms were separated by sex, counted and their length measured. Worms were then frozen at −20 °C for later DNA extraction.