Antibacterial Activity of Eggs and Egg Wax Covering of Selected Ixodid (Acari: Ixodidae) Ticks¹

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Abstract Antimicrobial activity of eggs and the wax covering of eggs has been observed in several tick (Acari) species, but any antimicrobial activity associated with ixodid (Acari: Ixodidae) tick eggs is unknown. The antimicrobial activity associated with eggs, wax extracts from eggs, and eggs from which wax was removed of the ixodids Haemaphysalis longicornis Neumann (parthenogenetic and bisexual populations), Haemaphysalis doenitzi Warburton et Nuttal, Dermacentor silvarum Olenev, and Hyalomma asiaticum Schulze and Schlottke was assessed using an *in vitro* agar diffusion assay. Eggs of *D. silvarum* and *Hy. asiaticum* placed on the agar culture inhibited the growth of gram-negative bacteria but had no activity against gram-positive bacteria. Eggs of H. longicornis (parthenogenesis and bisexual population) and H. doenitzi inhibited the growth of gram-positive bacteria, but not gram-negative bacteria. All eggs from which the wax had been extracted had no activity against either gram-positive or gram-negative bacteria. Organic (chloroform/methanol, 2:1) and aqueous extracts of wax from D. silvarum and Hy. asiaticum eggs inhibited the growth of gram-positive bacteria but had no inhibitory activity against gram-negative bacteria. Organic and aqueous extracts of wax from H. longicornis (parthenogenesis and bisexual population) and H. doenitzi eggs inhibited the growth of gram-negative bacteria, but not gram-positive bacteria. The diverse antimicrobial activities of the eggs and the wax extracted from the eggs of these tick species provide a basis for further study in the identification of novel therapeutic biomolecules.

Key Words Dermacentor silvarum, Hyalomma asiaticum, Haemaphysalis longicornis, Haemaphysalis doenitzi, antimicrobial activity

Ticks (Acari) are nonpermanent ectoparasitic arthropods of various terrestrial vertebrates and cause health problems directly through blood-feeding and indirectly as vectors of pathogens and toxins (Jongejan and Uilenberg 1994). Generally, the postembryonic stages of ixodid ticks (Acari: Ixodidae) feed for several days on their hosts and spend more than 90% of their lifetime off the host (Labruna et al. 2005). Engorged females detach and fall off their hosts in time to deposit their eggs (Anderson 2002). The large clutches of eggs produced by engorged females can survive for long periods until hatch. Even when the egg-laying females have been

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found dead and covered with fungi, the eggs next to the cadavers remain uninfected despite being in proximity to numerous naturally occurring microbes (Esteves et al. 2009, Potterat et al. 1997).

During oviposition, the newly laid eggs are coated with a waxy substance secreted by the Gene's organ (Booth 1992). The waxy substance has been recognized as preventing desiccation of eggs and acting as a physical barrier for chemical defense (Booth 1992). Only recently, the antimicrobial or antiviral activity of this substance was demonstrated in Amblyomma hebraeum Koch (Arrieta et al. 2006, Yu et al. 2012), Amblyomma cajennense (Fabricius) (Lima-Netto et al. 2011, 2012), Amblyomma aureolatum (Pallas), Rhipicephalus (Boophilus) microplus (Canestrini), and Rhipicephalus sanguineus (Latreille) (Alduini et al. 2014). However, any antimicrobial activity of the waxy substance associated with ixodid tick eggs has not been reported. Additionally, a variety of chemicals with antimicrobial activity have been identified from tick organs and tissues including the salivary glands (Liu et al. 2008, Pichu and Mather 2009, Zhou et al. 2007), the midgut (Belmonte et al. 2012, Nakajima et al. 2003), and hemolymph (Fogaca et al. 2006, Lai et al. 2004). Some of these molecules are of interest for development as novel pharmacological compounds (Alduini et al. 2014, Vizioli and Salzet 2002). Hence, the study reported herein investigated the antimicrobial potential of eggs, wax extracts from eggs, and eggs from which waxes were removed from several ixodid tick species. Results could serve as a foundation for further understanding of the function of wax from tick eggs and perhaps provide additional choices to identify and characterize novel therapeutic biomolecules.

Materials and Methods

Ticks. Dermacentor silvarum Olenev and the bisexual population of Haemaphysalis longicornis Neumann were collected from Zhangjiakou, Hebei (N 36°05′-42°40′, E 113°27′-119°50′). Haemaphysalis doenitzi Warburton et Nuttal and the parthenogenic population of H. longicornis were collected from Cangxi, Sichuan (N 31°37'-32°10', E 105°43'-106°28'). Hyalomma asiaticum Schulze and Schlottke was collected from Urumgi, Xinjiang (N 34°22'-49°33', E $73^{\circ}41'-96^{\circ}18'$). All ticks were collected by dragging a white flannel cloth (1.2 m long \times 1.0 m wide), stretched between two wooden rods, at a speed of 10 m/min for 500 m over vegetation. The clothes were checked every 20 m, and ticks attached to the cloth were collected, placed separately in 10-ml plastic tubes, and transported to the laboratory. After identification, the collected ticks were fed on rabbits. To facilitate feeding and management, ticks were placed into cloth bags attached by adhesive tape onto the ears of rabbits and checked daily (Liu et al. 2005). After engorgement, females were placed in separate 10-ml glass tubes for oviposition in a laboratory incubator maintained at 20 ± 1°C, 90% relative humidity, and a photoperiod of 16 h light, 8 h darkness. The use of rabbits for the purpose of this study was approved by the Animal Care and Use Committee of Hebei Normal University.

Egg treatments and wax extraction. Eggs were collected daily during the oviposition and stored at -20° C in a refrigerator until use in the assays. Wax was extracted from selected eggs of each tick species using a cholorform/methanol (v/

v = 2:1) and distilled water extraction method modified from Yu et al. (2012). Simply, 1 g of tick eggs was placed in 3 ml chloroform/methanol in a glass tube and vortexed for 15 s; the supernatant was transferred to a clean glass tube. Then, the eggs were immersed in 1 ml distilled water and vortexed; the supernatant was transferred and combined with the previous supernatant. The eggs were washed with cholorform/methanol (v/v = 2:1) again, and that supernatant was combined with the previous supernatant. The combined supernatant was centrifuged at 1400 ×g for 10 min to separate the phases. The upper phase (aqueous extract) was lyophilized and stored at -80° C, and the lower phase (organic extract) was dried under a nitrogen stream in a fume hood and stored at -20° C until ready for use. After extraction, the eggs from which the wax had been extracted were stored.

Assays of antimicrobial activity associated with eggs and extracted wax. An *in vitro* agar diffusion assay was used to determine the antimicrobial activity associated with wax extracts from the tick eggs, eggs from which the waxes had been extracted, and eggs that were not subjected to was extraction as previously described by Yu et al. (2012). Cultures of the gram-positive bacteria *Staphylococcus aureus* Rosenbach (ATCC25923), *Enterococcus faecalis* (Andrewes and Horder) Schleifer and Kilpper-Balz (ATCC981), *Enterococcus faecium* (Orla-Jensen) Schleifer and Kilpper-Balz (ATCC091299), and *Nocardia asteroides* (Eppinger) Blanchard (ATCC201118) and the gram-negative bacteria *Escherichia coli* (Migula) Castellani and Chalmers (ATCC25922), *Klebsiella pneumonia* (Schroeter) Trevisan (ATCC43816), *Salmonella enteritidis* (Gaertner) Castellani and Chalmers (ATCC13076), and *Pseudomonas aeruginosa* (Schroeter) Migula (ATCC47085) were cultured in Mueller–Hinton (MH) broth containing 1.5% agar. Each bacterium was mixed in 0.5% soft agar which was then poured on MH agar plates.

Eggs (no wax extraction or wax extracted) were placed directly on the agar surfaces overlaid with bacterial culture. The organic extract (10 mg dissolved in 50 μ l chloroform/methanol) or the aqueous extract (2 mg dissolved in 50 μ l sterilized water) was added to filter paper discs (6 mm), air dried for 15 min, and subsequently placed on the bacterial culture overlay plates. Plates were incubated for 48 h at 37°C, and the resultant inhibition zones were measured with calipers to the nearest 0.1 mm. Data were analyzed by analysis of variance using Statistica 6.0 software (Statsoft, Tulsa, OK).

Results

Wax extract quantity. No significant differences were observed in the total amount of wax extracted from the eggs, as well as the amounts within the organic and the aqueous phases of the extracts of the different tick species (F=7.19, df=4, P=0.41). Amounts of wax extracted are listed in Table 1.

Antibacterial activity of eggs. Eggs from which the waxes had been extracted showed no inhibitory effect against the bacteria tested in our study, regardless of the tick species. Eggs that had not undergone wax extraction, however, inhibited bacterial growth in these *in vitro* assays. *Haemaphysalis longicornis* (parthenogenic and bisexual populations) and *H. doenitzi* eggs inhibited the growth of gram-positive

Species	Organic phase*	Aqueous phase*	Total*
Dermacentor silvarum	17.9 ± 2.7	7.3 ± 4.1	25.2 ± 6.2
Hyalomma asiaticum	17.2 ± 4.4	7.1 ± 2.4	24.3 ± 4.8
Haemaphysalis longicornis (bisexual)	16.7 ± 4.1	7.5 ± 3.0	24.2 ± 6.2
Haemaphysalis longicornis (parthogenic)	16.8 ± 3.8	6.4 ± 1.9	23.2 ± 4.7
Haemaphysalis doenitzi	18.1 ± 3.9	7.0 ± 2.9	25.0 ± 2.7

Table 1. Mean $\pm\,$ SEM amounts (mg/g) of wax extracts from the eggs of selected ixodid ticks.

* No statistical differences within columns observed (P > 0.05).

bacteria, but not gram-negative bacteria (Table 2). On the other hand, eggs from *D. silvarum* and *Hy. asiaticum* inhibited the growth of gram-negative bacteria, but not gram-positive bacteria (Table 3).

Antibacterial activity of wax extracts. Both the organic and aqueous extracts of the waxes from *D. silvarum* and *Hy. asiaticum* eggs inhibited the growth of grampositive bacteria but not gram-negative bacteria (Table 2). And, the organic and aqueous extracts from *H. longicornis* (parthenogenic and bisexual populations) and *H. doenitzi* eggs inhibited the growth of gram-negative bacteria, but not grampositive bacteria (Table 3).

Discussion

These results demonstrate that the eggs of the ixodid ticks *H. longicornis* (parthenogenic and bisexual populations), *H. doenitzi, D. silvarum*, and *Hy. asiaticum* exhibit antibacterial activity. Furthermore, the waxes extracted from the eggs appear to contain the source of the antibacterial activity and the inhibitory effect may vary with the tick species. Our findings expand the information base on the antimicrobial activity of tick eggs and the waxes extracted from eggs. The eggs of *A. hebraeum* (Arrieta et al. 2006, Yu et al. 2012) and *R. microplus* (Esteves et al. 2009) also inhibited the growth of gram-positive bacteria, whereas waxes extracted from the eggs of *A. aureolatum* and *R. microplus* inhibited the growth of both gramnegative and gram-positive bacteria (Alduini et al. 2014). Wax extracts from the eggs of *R. microplus* also reportedly inhibit biofilm formation by both gram-negative and gram-positive bacteria (Zimmer et al. 2013), while wax extracts from *A. cajennense* eggs inhibit virus replication and growth of yeasts, fungi, and grampositive bacteria (Lima-Netto et al. 2011, 2012).

We postulate that antimicrobial activity associated with eggs of a variety of tick species is also a common characteristic among the ixodid ticks. Fatty acids with antimicrobial activity have been identified in the organic extract of waxes from *A. hebraeum* eggs, but antimicrobial factors in the aqueous extracts remain unknown (Yu et al. 2012). Additional efforts are required to further explore and characterize the inhibitory molecules located at the egg surface. However, our results add to our

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	n	imanipulated egg	#S	Organic e	xtract #	y snoənby	extract #
Tested bacteria	Haemaphysalis longicornis (bisexual)*	Haemaphysalis longicornis (parthogenic)*	Haemaphysalis doenitzř	Dermacentor silvarum*	Hyalomma asiaticum*	Dermacentor silvarum*	Hyalomma asiaticum*
Staphylococcus aureus	0.6 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	10.3 ± 0.8	10.3 ± 0.9	14.7 ± 0.5	14.7 ± 0.5
Enterococcus faecalis	0.5 ± 0.3	0.6 ± 0.3	0.4 ± 0.4	9.9 ± 0.7	10.5 ± 0.7	13.3 ± 0.9	13.9 ± 1.1
Enterococcus faecium	0.4 ± 0.2	0.4 ± 0.2	0.6 ± 0.2	10.1 ± 0.5	9.7 ± 0.5	15.1 ± 0.7	15.1 ± 0.7
Nocardia asteroides	0.3 ± 0.1	0.5 ± 0.1	0.7 ± 0.3	9.8 ± 0.5	10.2 ± 0.4	13.8 ± 1.1	13.4 ± 1.3

 $^{\#}$ Diameter of the clear zone includes diameter of paper discs (6 mm) and eggs. * No statistical difference was observed (P>0.05).

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	Unmanipula	ited eggs#		Organic extract [#]		4	vqueous extract	**
Tested bacteria	Dermacentor silvarum*	Hyalomma asiaticum*	Haemaphysalis Iongicornis (bisexual)*	Haemaphysalis longicornis (parthogenic)*	Haemaphysalis doenitzi*	Haemaphysalis longicornis (bisexual)*	Haemaphysalis longicornis (parthogenic)*	Haemaphysalis doenitzi*
Escherchia coli	0.6 ± 0.2	0.5 ± 0.1	10.9 ± 0.9	10.4 ± 0.9	10.5 ± 0.8	15.0 ± 0.5	15.0 ± 0.7	15.2 ± 0.4
Klebsiella pneumonia	0.7 ± 0.3	0.7 ± 0.3	10.8 ± 0.7	10.3 ± 0.7	9.8 ± 0.9	15.0 ± 0.5	13.6 ± 1.1	13.8 ± 1.2
Salmonella enteritidis	0.5 ± 0.2	0.5 ± 0.2	9.8 ± 0.5	9.9 ± 0.5	10.1 ± 0.4	14.6 ± 0.7	14.8 ± 0.5	15.3 ± 0.8
Pseudomonas aeruginosa	0.6 ± 0.2	0.6 ± 0.1	9.6 ± 0.4	9.7 ± 0.4	10.0 ± 0.3	13.5 ± 1.3	1 3.7 ± 1 .3	13.9 ± 1.2

Table 3. Mean ± SEM diameter of inhibition zone (mm) of the unmanipulated eggs and egg wax of selected ixodid ticks

 $^{\#}$ Diameter of the clear zone includes diameter of paper discs (6 mm) and eggs.

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understanding of the functions of waxy coverings on tick eggs and provide additional choices in developing potential therapeutic or pharmaceutical agents.

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