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Detection of human bacterial pathogens in ticks collected from Louisiana black bears (*Ursus americanus luteolus*)

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Abstract

There are 4 major human-biting tick species in the northeastern United States, which include: *Amblyomma americanum*, *Amblyomma maculatum*, *Dermacentor variabilis*, and *Ixodes scapularis*. The black bear is a large mammal that has been shown to be parasitized by all the aforementioned ticks. We investigated the bacterial infections in ticks collected from Louisiana black bears (*Ursus americanus* subspecies *luteolus*). Eighty-six ticks were collected from 17 black bears in Louisiana from June 2010 to March 2011. All 4 common human-biting tick species were represented. Each tick was subjected to polymerase chain reaction (PCR) targeting select bacterial pathogens and symbionts. Bacterial DNA was detected in 62% of ticks (n=53). *Rickettsia parkeri*, the causative agent of an emerging spotted fever group rickettsiosis, was identified in 66% of *A. maculatum*, 28% of *D. variabilis*, and 11% of *I. scapularis*. The Lyme disease bacterium, *Borrelia burgdorferi*, was detected in 2 *I. scapularis*, while one *Am. americanum* was positive for *Borrelia bissettii*, a putative human pathogen. The rickettsial endosymbionts *Candidatus* Rickettsia andeanae, rickettsial endosymbiont of *I. scapularis*, and *Rickettsia amblyommii* were detected in their common tick hosts at 21%, 39%, and 60%, respectively. All ticks were PCR-negative for *Anaplasma phagocytophilum*, *Ehrlichia* spp., and *Babesia microti*. This is the first reported detection of *R. parkeri* in vector ticks in Louisiana; we also report the novel association of *R. parkeri* with *I. scapularis*. Detection of both *R. parkeri* and *Bo. burgdorferi* in their respective vectors in Louisiana demands further investigation to determine potential for human exposure to these pathogens.

Introduction

Since Theobald Smith's seminal work describing ticks as the vectors for Texas cattle fever, the importance of arthropods as vectors for disease has surged to the forefront of both human and animal medicine (Kilborne, 1893). By the turn of the 20th century, many control measures were developed and deployed to combat the pests spreading these pathogens. Excellent reviews of these can be found in Hill et al. (2005) and Piesman and Eisen (2008). Even with advances in arthropod control and disease management, the world has seen an emergence and re-emergence of zoonotic vector-borne diseases within the past 30 years (Christou, 2011; Jones et al., 2008). Ticks are vectors for a plethora of zoonoses, which include the causative agents of anaplasmosis, babesiosis, ehrlichiosis, Lyme and relapsing

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fever borreliosis, spotted fever group (SFG) rickettsiosis, and a host of viral diseases (Dantas-Torres et al., 2012; Parola and Raoult, 2001). In the United States, ticks transmit more vector-borne diseases than any other arthropod. With over 80 known species of ticks in the US, opportunity exists for each one of the aforementioned diseases to affect humans and animals (Berrada and Telford, 2009).

Not only is the US witnessing a steady increase in reported human cases of many tick-borne diseases, new tick-borne pathogens are being discovered on a regular basis. For example in 1992, Lyme disease, Rocky Mountain spotted fever (RMSF), and tularemia were the only nationally notifiable tick-borne diseases; approximately 20 years later the number has swelled to nine (Anonymous, 2011). This increase amounts to 3 new tick-borne human diseases identified per decade. With the advent of better detection, diagnosis, and surveillance, this number is likely to increase.

Despite the increase in research on ticks and tick-borne diseases conducted elsewhere in the past several decades, comparatively less work has been done in Louisiana. With outdoor activity as the single most important risk factor for tick exposure (Piesman and Eisen, 2008) and Louisiana's recognition as "A Sportsman's Paradise", there is strong risk of tick exposure for both residents and visitors. In the southern US, humans are most commonly parasitized by 4 ticks species: *Amblyomma americanum*, *Amblyomma maculatum*, *Dermacentor variabilis*, and *Ixodes scapularis* (Goddard, 2002; Merten and Durden, 2000; Felz et al., 1996). Each of these tick species have unique host preferences that, taken together, encompasses a range of host animals (Oliver, 1989). Black bears have been reported to harbor many of the same tick species as humans, and not surprisingly, pathogenic bacteria have been detected in ticks parasitizing bears (King, 1960; Rogers and Rogers, 1976; Binninger et al., 1980; Schroeder, 1987; Kazmierczak et al., 1988; Yabsley et al., 2009; Nims and Durden, 2011).

Louisiana's state animal is the black bear, *Ursus americanus* subspecies *luteolus*, which has been listed as a threatened species by the United States Fish and Wildlife Service since 1992. The Louisiana Department of Wildlife and Fisheries carefully monitors bear populations throughout the state and has collected ectoparasites during these monitoring efforts. The objective of our study was to investigate which ticks parasitize black bears in Louisiana and to identify bacteria of interest that they harbor.

Materials and methods

Bear sampling and tick collection

From June 2010 to March 2011, ticks were collected from 17 black bears throughout Louisiana by personnel from Louisiana's Department of Wildlife and Fisheries. Ticks collected off bears in this study were convenience samples, as the bears consisted of relocated and accidentally or illegally killed animals encountered by the Department of Wildlife and Fisheries personnel. Bears were sampled from 7 parishes: Concordia, Iberville, Livingston, Madison, Pointe Coupee, St. Landry, and St. Mary. Exhaustive searching of bears for ectoparasites was not conducted, leading to the collection of only easily visible adult ticks. Ticks were stored in 70% ethanol for transport, then identified and sexed using standard identification keys (Sonenshine, 1979; Diamant and Strickland, 1965).

Tick DNA extraction

Ticks were surface-sterilized by first soaking in a 10% bleach solution, followed by a phosphate buffered saline (PBS) wash, next soaked in a 70% ethanol solution, followed by a PBS wash and finally a soak in molecular biology grade water (MO BIO, Carlsbad, CA).

Individual ticks were then bisected with a sterile scalpel, and DNA was extracted from each tick using the GenElute DNA extraction Kit (Sigma, St. Louis, MO) following manufacturer's instructions with minor modifications. These modifications included: Tick tissues were allowed to lyse overnight in a 55°C water bath, and DNA was eluted in 100 μl of supplied elution buffer. DNA was stored at −20°C until PCR was performed.

Polymerase chain reaction

Polymerase chain reaction (PCR) was performed on DNA extracts to detect the presence of selected bacteria. The PCR assays targeted the following genes: *msp2* for *Anaplasma phagocytophilum* (Caspersen et al., 2002), 18s rRNA for *Babesia microti* (Persing et al., 1992), *flaB* for *Borrelia burgdorferi* sensu lato (Clark et al., 2005), 16s rRNA for *Ehrlichia* species (Anderson et al., 1992; Dawson et al., 1994), and both 17-kDa antigen gene (Carl et al., 1990) and *ompA* (Regnery et al., 1991) for SFG rickettsiae. Environmental controls were utilized for detection of DNA artifact contamination in all experiments. This included simultaneous extraction tubes with no tissue added for all DNA extractions and molecular biology grade water (MO BIO) used as a template in the downstream PCRs. Initial reactions contained 2.5 μL of DNA extract in a total reaction volume of 25 μL. TaKaRa Taq DNA polymerase (TaKaRa-Bio Inc, Otsu, Japan) was utilized in all reactions. Each reaction had a final concentration of 1.25 U of Taq DNA polymerase, 45 mM KCL, 2.5 mM $MgCl_2$, 200 μM concentration of each deoxynucleoside, and 0.5 μM of the upstream and downstream primers. All reactions were carried out in a My Cycler thermal cycler (BioRad, Hercules, CA). Nested reactions contained 1 μL of product from outer reaction under the same master mix concentrations. Thermocycling protocols were followed as published for each primer set.

DNA purification/sequencing/analysis

PCR products were electrophoresed on a 2% agarose gel and stained with ethidium bromide. Bands of the correct size were purified with a QIAquick PCR purification kit (Qiagen, St. Louis, MO) and bi-directionally sequenced on an automated ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA) at LSU's Biomed Gene Lab. Sequences were aligned using ClustalX and compared with existing sequences in the National Center for Biotechnology Information (NCBI) GenBank using the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990; Thompson et al., 1997).

PCR cloning

PCR-cloning was utilized to identify amplicon inserts in samples where multiple signals were detected in the sequencing chromatograph. Cloning reactions were performed according to manufacturer's instructions with an Invitrogen Topo TA cloning kit (Life Technologies, Grand Island, NY). Multiple colonies were picked, and the universal primers M13F and M13R were used to sequence as previously described. The resulting sequences were aligned and analyzed as described above.

Results

Four tick species were found parasitizing Louisiana black bears

Eighty-six ticks were removed from a total of 17 bears. All ticks collected were adults; 51% (n=44) were females and 49% (n=42) were males. *Am. maculatum* was the most commonly collected tick, comprising 44% of all ticks collected (n=38), *D. variabilis* represented 29% (n=18) of the total collected, *I. scapularis* was the next most abundant at 21% (n=15), and the least common was *Am. americanum* at 6% (n=5) (Table 1).

Three bacterial pathogens were detected in ticks

DNA was extracted from all 86 ticks collected from Louisiana black bears. These extracts were tested for the presence of *An. phagocytophilum*, *Bo. burgdorferi* sensu lato, *Ba. microti*, *Ehrlichia* spp., and SFG *Rickettia* DNA by PCR. Pathogenic bacterial DNA was detected in 44% (n=38) of all ticks. *Rickettsia parkeri* was detected most frequently with 25 of 38 (65.7%) *Am. maculatum*, 7 of 25 (28%) *D. variabilis*, and 3 of 18 (11.1%) *I. scapularis* testing positive. *R. parkeri* sequences were 99% homologous with sequences in GenBank (Table 2). Evidence of *Bo. burgdorferi* sensu stricto was found in 2 of 18 (11%) *I. scapularis*, while one (20%) *Am. americanum* had a sequence matching *Bo. bissettii* (Table 2). No *An. phagocytophilum*, *Ba. microti* or *Ehrlichia* species DNA were detected in any of the ticks.

Three rickettsial endosymbionts were detected in ticks

SFG *Rickettsia* primers and subsequent sequencing identified rickettsial endosymbiont DNA in 20.9% (n=18) of their respective ticks. These endosymbionts included: *Candidatus* Rickettsia andeanae in 8 of 38 (21%) *Am. maculatum*, rickettsial endosymbiont of *I. scapularis* in 7 of 18 (38.8%) *I. scapularis*, and *Rickettsia amblyommii* in 3 of 5 (60%) *Am. americanum*. Endosymbionts were 99–100% similar to their respective reference sequences in GenBank (Table 2).

Multiple bacteria detected in ticks

Ticks with evidence of mixed SFG *Rickettsia* DNA after sequencing were subjected to PCR cloning and sequencing of multiple clones to identify individual amplicons. Two ticks had the presence of multiple SFG *Rickettsia* DNA. This included one *Am. maculatum* with both *Candidatus* R. andeanae and *R. parkeri*, and one *I. scapularis* with the rickettsial endosymbiont of *I. scapularis* and *R. parkeri*. Two additional ticks had evidence of multiple bacteria by separate PCRs. In one *I. scapularis*, we detected both *B. burgdorferi* and the rickettsial endosymbiont of *I. scapularis*, and one *Am. americanum* had evidence of both *R. amblyommii* and *Bo. bissettii* DNA (Table 2).

Discussion

In this study, we investigated which species of ticks were parasitizing Louisiana black bears and identified both pathogenic and symbiontic bacterial DNA in the arthropods. Human-biting ticks have been commonly recorded on bears in studies across the US dating back to the mid 1970s (Nims and Durden, 2011; Rogers and Rogers, 1976; Rogers, 1975; King, 1960; Yabsley et al., 2009). A previous study in Idaho detected antibodies to multiple tick-borne pathogens in black bears whilst a Wisconsin study isolated Lyme *Borrelia* spirochetes from these animals (Kazmierczak et al., 1988; Binninger et al., 1980). More recently, Yabsley et al. (2009) detected *Ehrlichia chaffeensis* and *R. parkeri* in ticks from black bears in southern Georgia/northern Florida. Consistent with these previous reports, black bears in Louisiana harbor many common human-biting ticks; nearly half of these had PCR evidence of bacterial pathogens.

Most importantly, this is the first record of *R. parkeri* infection occurring in its natural vector in Louisiana, with almost 66% of the *Am. maculatum* we tested having evidence of *R. parkeri*. This coincides with a recent publication detecting *R. parkeri* in the blood of dogs in Louisiana (Grasperge et al., 2012). *R. parkeri* rickettsiosis is an emerging infectious disease. Detection of *R. parkeri* is becoming more common across the range of its primary vector, *Am. maculatum*. Published infection rates of *R. parkeri* range from ~10 to 40% in populations of southeastern *Am. maculatum* ticks (Varela-Stokes et al., 2011; Ferrari et al., 2012; Fornadel et al., 2011; Paddock et al., 2010; Sumner et al., 2007). Multiple ticks from

multiple parishes in Louisiana had evidence of *R. parkeri* infection (Fig. 1). *R. parkeri* was also detected in both *D. variabilis* and *I. scapularis*. This is the first record of detection of this SFG *Rickettsia* in *I. scapularis* ticks. These ticks are not considered vectors for *R. parkeri*, but are known to harbor other SFG *Rickettsia*. Interestingly, we detected *R. parkeri* in 2 male *I. scapularis*, considering male *Ixodes* ticks feed sparingly if at all, this finding raises the question of pathogenic SFG *Ricksttia* infection in *I. scapularis* (Kiszewski et al., 2001). Identifying such a large number of *R. parkeri*-infected ticks associated with bears also proposes the question of the bear as a potential reservoir host. Lyme *Borrelia* spirochetes were detected in 3 ticks. *Bo. burgdorferi* sensu stricto was detected in 2 *I. scapularis* while *Bo. bissettii* was detected in a male *A. americanum*. *Bo. burgdorferi* sensu stricto is the leading cause of the most common vector-borne disease in the US, Lyme disease. *Bo. bissettii* is a closely related Lyme spirochete that shares common vectors, hosts, and geographic range and more recently has been implicated in human disease (Girard et al., 2011; Rudenko et al., 2008, 2009).

Lack of detection of *Ba. microti*, *An. phagocytophilum*, or *Ehrlichia* species should not be taken as evidence of their non-existence in Louisiana, as information regarding ticks and their associated diseases in Louisiana is still lacking. Surrounding states like Arkansas, Mississippi, and Texas host a range of tick-borne diseases that have been determined only after large samples and years of research (Castellaw et al., 2010; Williamson et al., 2010; Fryxell et al., 2012; Goddard et al., 2003).

Many species of ticks are associated with distinct endosymbiontic bacteria (Rounds et al., 2012). In this study, we detected multiple symbiontic SFG *Rickettsia*. *Candidatus* R. andeanae is a recently isolated SFG *Rickettsia* and a putative endosymbiont of *Am. maculatum* ticks (Luce-Fedrow et al., 2012). It has been reported from multiple regions of the US where *Am. maculatum* are found (Ferrari et al., 2012; Fornadel et al., 2011; Jiang et al., 2012). Our finding of *Candidatus* R. andeanae in 21% of *Am. maculatum* is the first evidence of this bacterium in Louisiana and expands the known range of this recently described SFG *Rickettsia*. Detection of other SFG rickettsial endosymbionts, namely *R. amblyommii* in *Am. americanum* and the rickettsial endosymbiont of *I. scapularis*, is not unexpected, and these are the first published records of these SFG rickettsiae in Louisiana ticks. Anecdotal reports have implicated *R. amblyommii* in human disease, but the majority of these conclusions have yet to be substantiated in laboratory studies (Smith et al., 2010; Billeter et al., 2007; Apperson et al., 2008; Nicholson et al., 2009; Jiang et al., 2010).

This study was conducted retrospectively on ticks collected from bears by the Louisiana Department of Wildlife and Fisheries personnel. Due to various limitations, the collected ticks probably do not comprise the total tick population on each bear. It is expected that immature stages of ticks were overlooked during sampling, as ectoparasite collection was not the focus of the Fish and Wildlife personnel's work. In addition, many of the female ticks collected and subjected to PCR were at different stages of engorgement, yet others had no obvious signs that a blood meal had begun. These limitations directly affect the interpretation of the data presented in that, while it is possible that detection of bacteria in these ticks may indicate infection, it is just as likely that these bacteria were imbibed during feeding on the host bear either by co-feeding transmission or by an infection in the bear. Future studies should ascertain infection status in questing, unfed, ticks in Louisiana.

With the resurgence of zoonotic vector-borne diseases in the past 3 decades, the importance of disease surveillance and control has become apparent. In the US, the recent discoveries of tick-borne zoonotic pathogens like *R. parkeri*, *Rickettsia* spp. 364D, *Ehrlichia muris*-like (EML), Panola mountain *Ehrlichia*, and Powassan virus combined with the increasing frequency of tick-borne diseases like Lyme disease, SFG rickettsiosis, anaplasmosis,

ehrlichiosis, and babesiosis have more than proven a need to closely monitor tick-borne diseases (Shapiro et al., 2010; Reeves et al., 2008; Pritt et al., 2011; Paddock, 2009). Since little is known about the organisms circulating in Louisiana ticks, detection of bacteria in ticks that commonly parasitize humans improves our understanding of tick-borne disease risks to human and animals in the state. The detection of *R. parkeri* and *Bo. burgdorferi* and the novel association of *R. parkeri* with *I. scapularis* in ticks collected from black bears prompts more questions on the prevalence of tick-borne pathogens in questing and other host-associated ticks across Louisiana. Surveillance of pathogens in human-biting ticks in Louisiana is essential to educating health professionals, elucidating tick-borne disease risk, and ultimately protecting the health of the public.

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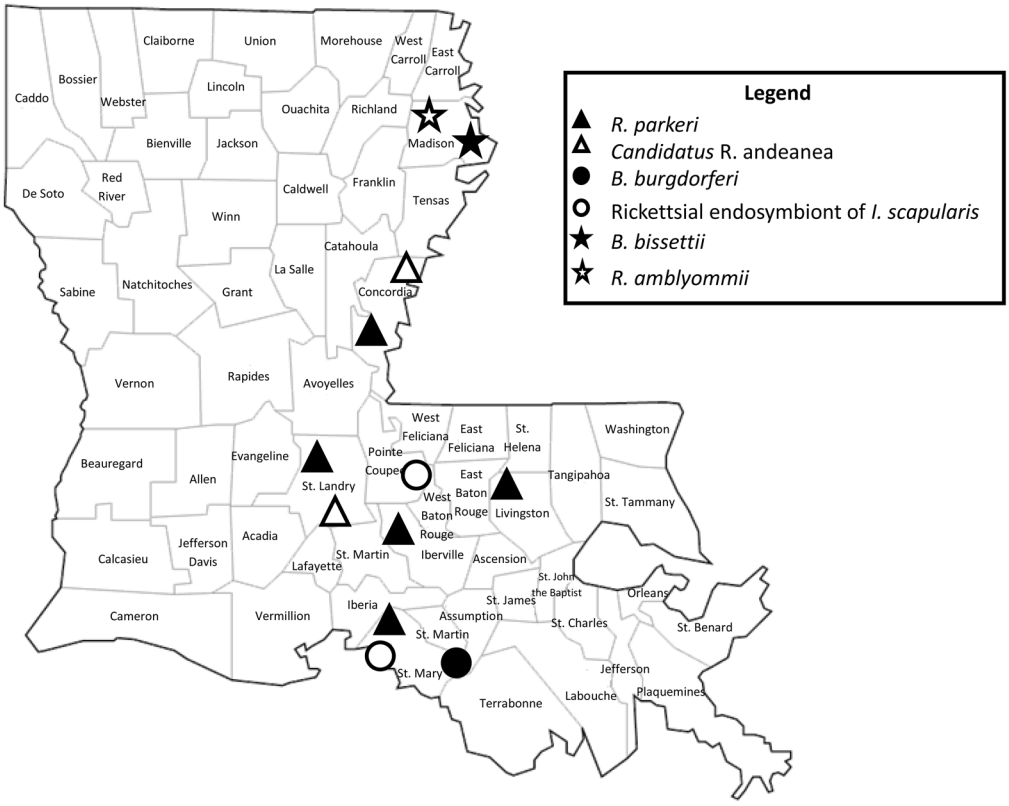

Fig. 1.
Location of ticks positive for bacterial DNA tested for in this study.

Table 1

Number, month sampled, species, and gender of ticks (M=male, F=female) collected from each of the bears in this study.

Bear	Month sampled	*Amblyomma americanum*	*Amblyomma maculatum*	*Dermacentor variabilis*	*Ixodes scapularis*	Total
1	June	–	4 (M), 4 (F)	1 (M)	–	9
2	June	–	2 (M), 4 (F)	1 (F)	–	7
3	June	–	–	2 (M), 1 (F)	–	3
4	June	–	9 (M)	2 (M), 3 (F)	–	14
5	June	–	–	1 (M), 6 (F)	–	7
6	June	–	3 (M), 1 (F)	1 (M), 3 (F)	–	8
7	September	–	–	1 (F)	–	1
8	September	–	–	1 (F)	–	1
9	September	–	1 (M)	1 (M), 1 (F)	–	3
10	October	–	9 (M), 1 (F)	–	–	10
11	October	–	–	–	2 (M), 3 (F)	5
12	November	–	–	–	2 (M), 1 (F)	3
13	December	–	–	–	2 (F)	2
14	January	–	–	–	2 (F)	2
15	February	–	–	–	2 (F)	2
16	February	3 (M), 2 (F)	–	–	–	5
17	March	–	–	–	2 (M), 2 (F)	4
Total		**5**	**38**	**25**	**18**	**86**

Table 2

Tick DNA samples PCR-positive in this study. Identical sequences are represented by identical accession numbers. Multiple infected samples are denoted by asterisks.

Sample ID	Tick species	Accession number	Homology
S1AM1	*Amblyomma maculatum*	KC003477	99% *Rickettsia parkeri*
S1AM3, S1AM4, S1AM5, S1AM6, S1AM8	*Am. maculatum*	KC003475	100% *Candidatus* R. andeanae
S2AM3*	*Am. maculatum*	KC003475	100% *Candidatus* R. andeanae
S2AM3*	*Am. maculatum*	KC003476	99% *R. parkeri*
S2AM1, S2AM2, S2AM4, S2AM5, S2AM6	*Am. maculatum*	KC003477	99% *R. parkeri*
S3DV1, S3DV2	*Dermacentor variabilis*	KC003477	99% *R. parkeri*
S4AM1, S4AM2, S4AM3, S4AM, S4AM6, S4AM7, S4AM8, S4AM9	*Am. maculatum*	KC003477	99% *R. parkeri*
S4DV1, S4DV2, S4DV3	*D. variabilis*	KC003477	99% *R. parkeri*
S5DV3	*D. variabilis*	KC003477	99% *R. parkeri*
S6AM1, S6AM3	*Am. maculatum*	KC003477	99% *R. parkeri*
S6DV1	*D. variabilis*	KC003477	99% *R. parkeri*
S10AM1, S10AM2, S10AM3, S10AM4, S10AM5, S10AM6, S10AM7, S10AM8, S10AM9, S10AM10	*Am. maculatum*	KC003477	99% *R. parkeri*
S10AM5, S10AM6	*Am. maculatum*	KC003475	100% *Candidatus* R. andeanae
S11IS3*	*Ixodes scapularis*	KC003478	99% *R. parkeri*
S11IS2, S11IS3*, S11IS4	*I. scapularis*	KC003474	100% rickettsial endosymbiont of *I. scapularis*
S12IS1, S12IS2*	*I. scapularis*	KC003478	99% *R. parkeri*
S12IS2*	*I. scapularis*	KC003471	99% *Borrelia burgdorferi*
S13IS1	*I. scapularis*	KC003470	99% *Bo. burgdorferi*
S14IS2	*I. scapularis*	KC003472	100% rickettsial endosymbiont of *I. scapularis*
S16AA1, S16AA2*, S16AA4	*Am. americanum*	KC003473	99% *R. amblyommii*
S16AA2*	*I. scapularis*	KC003469	99% *Bo. bissettii*
S17IS4	*I. scapularis*	KC003472	100% rickettsial endosymbiont of *I. scapularis*
S17IS3	*I. scapularis*	KC003474	100% rickettsial endosymbiont of *I. scapularis*