Salivary Gland Thrombostasin Isoforms Differentially Regulate Blood Uptake of Horn Flies Fed on Control- and Thrombostasin-Vaccinated Cattle

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J. Med. Entomol. 47(4): 610–617 (2010); DOI: 10.1603/ME09251

ABSTRACT Thrombostasin (TS) is an anticlotting protein found in saliva of *Haematobia irritans* (horn flies). The polymorphic nature of the *ts* gene was first associated with success of horn flies blood feeding on a laboratory host, New Zealand White rabbits. In this study, we report results of similar studies testing blood uptake of horn flies feeding on a natural host, cattle. These studies confirmed the association of *ts* genotype with blood uptake of horn flies and showed that it was host species specific. In contrast to rabbits, blood uptake volumes of homozygous *ts10* horn flies were lower than those of other *ts* genotypes when fed on control (ovalbumin-vaccinated) cattle. Cattle vaccinated with recombinant protein isoforms, rTS9 or rTB8, resisted horn fly feeding by yielding lower blood volumes compared with flies feeding on control cattle. The specific impact of vaccination, however, varied by *ts* genotype of flies. Cattle vaccinated with isoform rTS9 resisted flies of *ts2*, *ts9*, and *tb8* genotype. Vaccination with isoform rTB8 produced resistance to *ts8*, *ts9*, and *tb8* genotype flies. Horn flies of genotype *ts10* were not affected by vaccination with either TS isoform and fed as well on rTS9- and rTB8-vaccinated as on control-vaccinated cattle. These experimental results confirm the efficacy of vaccines targeting horn fly salivary proteins and provide new insight into the dynamics of horn fly-cattle interactions in nature.

KEY WORDS horn fly, thrombostasin, blood feeding, cattle, vaccine

Parasitism by blood-feeding horn flies, *Haematobia irritans irritans* (L.), exacts a toll on cattle health and well being, which results in economic losses estimated to approach $1 billion in North America alone (Cupp et al. 1998). In addition to the direct physiological impact of their feeding on cattle, horn flies are known to transmit the bovine filarial parasite, *Stephanofilaria stilesi* (Hibler 1966), and the causative agent of bovine mastitis, *Staphylococcus aureus* (Owens et al. 1998, 2002). The benefits of horn fly control for promoting animal health and productivity have been demonstrated using presently available chemical means (Derouen et al. 2009, Sanson et al. 2003, Guglielmone et al. 1999).

Classical methods of chemical control, however, typically lead to selection for insecticide resistance that can severely limit the lifetime of any particular formulation. These complications of insecticidal use highlight the need to find other, more specific and long lasting, means of disrupting *H. irritans* parasitism of cattle (Oyarzún et al. 2008). Basic studies of horn fly blood feeding revealed the importance of salivary proteins in fly-cattle interactions that lead to successful parasitism (Cupp et al. 1998). These studies identified a dominant thrombin-inhibiting protein, thrombostasin (TS), in horn fly saliva and implicated it as a key factor in successful blood feeding by this important ectoparasite of cattle (Cupp et al. 2000).

DNA analyses of *ts* genes in horn flies, collected from field populations and from a colonized strain, uncovered multiple point mutations at fixed positions (Zhang et al. 2001). Two mutations were silent, whereas the remaining five specified peptides differ in molecular weight, isoelectric point, and predicted secondary structure. Comparative analysis of the allelic mutations and their predicted effects on secondary

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0022-2585/10/0610–0617$04.00/0

N_tag_TB8
*MRGSHHHHHHGMASMTGGQQMGRDLYDDDDKDP***QNLVSGRRQHGAQGLSGY**
SGDNDWGYYGEAGAPGSDYSGSSGQWAPLDFDYNSLPGLSGYNHEQQDYE
EDSYRHVRSAGPITLQLDDDDDDDDSGIPIFEMDDED[V]DSNDNQKFPLSFERF
PENEKNQ[V]GLRARFNKFMAKFTSLFGRRRGV[N]VPNAA

C_tag_TS9
*MA***QNVLSGRRQHGAQGLSGYSGDNDWGYYGEAGAPGSDYSGSSGQWAPLD**
FDYNSLPGLSGYNHEQQDYEEDSYRHVRSAGPITLQLDDDDDDDDSGIPIFEM
DDED[E]DSNDNQKFPLSFERFPENEKNQ[E]GLRARFNKFM AKFTSLFGRR
RGV[D]VPNAA*QLYTRASQPELAPEDPEDLEHHHHHHHH*

Fig. 1. Recombinant horn fly salivary vaccine protein constructs. Amino acids shown in bold comprise the native salivary proteins, whereas those in italics derive from the vector tag. Underlined amino acids identify the portion of TS that comprises the processed, active antithrombin portion of the two isoform proteins. Amino acids enclosed in brackets identify the single amino acid variants of the isoforms.

structure of the active proteins suggested that evolutionary selection may be acting on the TS gene in response to one or more environmental pressures. This analysis implied that the selected changes in TS structure might enhance action of TS in thrombin inhibition and/or might diminish negative host immune responses that neutralize TS action (Zhang et al. 2001).

Recently published studies examined the possibility of a relationship between the volume of blood obtained by a horn fly when fed on a laboratory host, New Zealand White (NZW) rabbits, and the fly's gender or its *ts* genotype (Cupp el al. 2009). These studies found that blood uptake was not related to horn fly gender, but was correlated with *ts* genotype. When physiologically similar flies of mixed gender and genotype were fed as a cohort on the same rabbits, blood volumes were diminished for those flies carrying even a single *ts9* allele. In contrast, inheritance of one or both *ts10* alleles was associated with increased blood-feeding volumes. Neither the *tb8* nor the *ts2* alleles were associated with a significant impact on blood feeding of NZW rabbits. Flies with the fifth most prominent *ts* allele (*ts8*) were too rare in the experimental population to provide meaningful analysis.

In studies reported in this work, we looked for similar relationships between a horn fly's *ts* genotype and blood-feeding volumes obtained when fed on its natural host, cattle. These studies were part of a larger project investigating the effects of cattle vaccination against salivary TS on horn fly-cattle interactions. Control-vaccinated cattle were used as a model to assess the impact of *ts* genotype on flies feeding on similar cattle (mixed breed dairy cattle, primarily Holstein) in the field. Cattle vaccinated with rTB8 or rTS9 were used to evaluate the potential of a TS vaccine to disrupt horn fly feeding and to assess any potential effects on vaccine efficacy corresponding to horn fly genotype.

Materials and Methods

Data for the horn fly blood-feeding and genotype analyses presented in this study were gathered as part of two cattle vaccine trials conducted 2 yr apart in 2002 and 2004. Cattle were vaccinated with ovalbumin (OVA; control) or rTB8 proteins in the first trial and with OVA (control), rTB8, or rTS9 proteins in the second trial. Overall blood-feeding results of the 2002 trial were presented previously (Cupp et al. 2004), and similar results for the 2004 trial are contained in this report. A subset of flies from the two trials was analyzed further for specific *ts* genotype (438 of 798 total flies) and tested for association with blood-feeding success.

Preparation of Vaccine Antigens. Preparation of rTB8, the first recombinant isoform produced for a vaccination trial, was described previously (Cupp et al. 2004). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis of rTB8 showed that, after expression in *Escherichia coli* and purification, five related peptides were recovered with the most abundant peptide lacking 41 amino acids from the C′ terminus (Cupp et al. 2004). All recovered rTB8 peptides, including a full-length peptide, were combined for the vaccine, which was subsequently labeled TSPool. The same rTB8 protein used in that vaccine trial was used in the second trial conducted in 2004.

An additional isoform, rTS9, was prepared for use in the second trial to compare efficacy with rTB8. Recombinant TS9 (rTS9) vaccine was prepared from a cDNA isolated from a horn fly salivary gland library (Zhang et al. 2001) after subcloning into the pTriEx-4 vector (Novagen) for expression in *E. coli* Rosseta strain host cells. The rTS9 fusion protein, with a purification tag at the C′ terminus, was extracted from the soluble fraction and purified with a HIS-binding resin (His.Bind, Novagen, Madison, WI). rTS9 was purified further with reversed phase high performance liquid chromatography. Analysis by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western blotting showed a peptide pool, similar to rTB8, which was >95% pure (data not shown). Endotoxin was removed from the newly prepared vaccine using EndoTrap Blue (Boca Scientific, Boca Raton, FL), according to manufacturer's instructions.

Amino acid composition of the two recombinant TS proteins used for vaccination are shown in Fig. 1. The three sites where amino acid variants occur in the active, mature antithrombin proteins are enclosed in brackets. Vaccine protein constructs also differed in the location of vector-added elements (identified by italics), including a HIS purification tag, which were

Table 1. Immunization schedule of second vaccine trial

Cattle group (n = 3 calves)	Prime	Boost 1	Boost 2	Boost 3
1	day 0	day 14	day 35	–
2	day 0	day 14	day 35	day 108
3	day 0	day 14	day 35	day 77
4	day 0	day 14	day 62	–

located on the N′ terminus for rTB8 and on the C′ terminus for TS9 (Fig. 1).

Cattle Host. Housing and vaccination of horn fly-naive, mixed-breed male dairy calves have been described in detail for one of the two cattle vaccine trials providing data for this report (Cupp et al. 2004). Calf characteristics, housing, and vaccination procedures were similar for the second trial and are described briefly below. All animal-related protocols were approved by the Auburn University Institutional Animal Care and Use Committee.

Immunization. Six calves were tested in vaccine trial 1, and 12 were used for trial 2. For both experiments, calves were born in winter when no adult horn fly exposure would occur, age matched, and placed in groups for immunization and testing. Details of trial 1, with three groups of rTB8- or OVA (control)-immunized calves, have been reported previously (Cupp et al. 2004). For the second trial, 12 calves were placed into one of four groups of three. One calve within each group was immunized with OVA (control), rTB8, or rTS9 antigen proteins using the regimen shown in Table 1. Blood uptake trials were conducted for calves from all four groups, whereas *ts* genotype was determined for flies exposed to calves in groups 1 and 2.

Vaccine emulsions were formed with 50 μg of antigen protein in Freund's complete adjuvant for the priming dose, and with Freund's incomplete adjuvant for the boosts. The priming dose was subdivided into four portions that were injected intradermally and subcutaneously. Boosting injections were all subcutaneous. Immunizations were administered, and testing of cattle was conducted by group to avoid any unpredictable environmental bias for a specific treatment.

Host Response to Immunization. Extent and specificity of serum antibody response of individual cattle were measured by enzyme-linked immunosorbent assay to confirm an active immune response. Microtiter wells were coated with 5 μg of test protein, and antibody binding from sera diluted 500- to 512,000-fold was tested. Detection of specific binding used peroxidase-labeled goat anti-bovine immunoglobulin G and tetramethylbenzidine peroxidase substrate (K&P Laboratories, Gaithersburg, MD), with color intensity measured by absorption at 450 nm. Additional effects of vaccination on calf cellular immune response and fly reproduction have been described for trial 1 (Cupp et al. 2004), and additional details will be described for the second trial in a subsequent report (M.S.C., E.W.C., C. N., D. Z., L. T., and V. P.; unpublished data).

Horn Flies. Horn fly pupae were shipped overnight from a colony maintained by the United States Department of Agriculture Livestock Insects Laboratory (Kerrville, TX). Immediately upon arrival, they were stored at 4°C to attenuate further development until needed for experimental use. For adult development, pupae were gently mixed for uniformity before a portion was transferred to an open petri dish within an emergence cage (constructed from a cardboard ice cream carton), and placed in an insect incubator at 28°C with a photoperiod of 16:8 (L:D) h. A water-soaked cotton ball, placed on the top screen of the cage, provided moisture during emergence of metabolically similar adult flies of either sex.

Blood Uptake Studies. Blood uptake of horn flies from control- and TS-vaccinated calves was assessed using quantitative measurement of individual blood meals. A feeding cage (7.5 cm in diameter × 1.5 cm deep) was secured to the hide on the back of the calf by three sutures. Twenty flies representing a mixture of sexes were added to the preattached cage and allowed to feed for exactly 20 min before the sutures were clipped, and the cage was removed from the calf's back. Blood digestion was inhibited by placing the feeding cage with flies in a cooler on a layer of paper towels underlain with crushed ice. Individually dissected mid-guts were added to an aliquot of Drabkins reagent, which forms a cyanide-hemoglobin complex that absorbs light of 540 nm wavelength (Briegel et al. 1979). Blood volumes were determined from a standard curve prepared from cattle host blood, which was drawn immediately after fly feeding (Cupp et al. 2004).

Genomic Analyses. A randomly selected subset of frozen carcasses of flies previously evaluated for blood feeding on control- or TB8-vaccinated calves (Cupp et al. 2004) was analyzed for *ts* genotype as part of the data set for this report. Additionally, a subset of horn flies from the second TS vaccination study, conducted in 2004 and described above, was evaluated to provide the remaining observations. From a total of 798 horn flies evaluated for blood uptake in those two studies, a total of 438 was evaluated further for *ts* genotype and comprises the data set for this report.

Immediately after blood meal removal, the fly carcass was placed in 500 μl of absolute ethanol and frozen at −70°C for subsequent DNA extraction. Total genomic DNA of individual horn flies was extracted using a method described previously (Zhang et al. 2001). In brief, individual flies were homogenized with 40 μl of buffer (10 mM Tris-HCl, pH 8.0, 2 mM ethylenediaminetetraacetic acid, and 0.4 M NaCl), and total DNA was extracted from the homogenate. Ten to 50 ng of DNA was used from each fly for polymerase chain reaction with *Taq*DNA polymerase. The following primer pairs were used: HITS8 (5′-ATCATGAAGCAT TTCGTAG-3′) and HITS18 (5′-GCT TAT GCA GCA TTG GGA ACA-3′). The polymerase chain reaction was carried out by mixing the following components in a final volume of 50 μl: 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM dNTPs, and 0.2 μM each primer and genomic DNA. The mixture was incubated at 94°C for 3 min and then maintained at 80°C until 2.5 U of polymerase was added to each reaction. Amplification for 35 cycles was

Table 2. Antibody response measured by enzyme-linked immunosorbent assay (serum dilution of 1/1000)

Sera from calf vaccinated with	OD 450 (vaccine antigen)	OD 450 (test TB8)	OD 450 (test TS9)
OVA	0.49 ± 0.28	0.13 ± 0.03	0.06 ± 0.02
TB8	0.64 ± 0.31	0.50 ± 0.08	0.27 ± 0.11
TS9	0.59 ± 0.17	0.67 ± 0.21	0.61 ± 0.32

OD, optical density.

followed: 94°C for 45 s, 60°C for 45 s, and 72°C for 80 s. A final extension step was carried out at 72°C for 7 min. Amplification products were directly sequenced after separation by agarose gel and purification with Sephaglas BP (Pharmacia Biotech, Uppsala, Sweden). Sequencing chromatograms and digital readout files of the amplification products were generated by the Auburn University Genetics Analysis Laboratory. Further nucleotide and amino acid analyses were conducted using Vector NTI program version 9. TS gene allele assignment was performed, as described previously (Zhang et al. 2001).

Statistical Analysis. Data were evaluated using Systat Software, version 11.0.0.1 (Systat Software, San Jose, CA).

Results

Calves responded to immunization with an increase in specific antibody to their vaccine antigen (Table 2, column 2). OVA-immunized (control) cattle showed serum antibody recognition of OVA (Table 2, row 2, column 2), but only background response to rTB8 (Table 2, row 2, column 3) or rTS9 (Table 2, row 2, column 4). TB8-immunized calves had serum antibody that bound rTB8 protein 2-fold greater than rTS9 (Table 2, row 3, columns 3 and 4), whereas antibody generated in TS9-immunized calves bound rTS9 and rTB8 equally well (Table 2, row 4, columns 3 and 4).

From the set of all flies evaluated for blood uptake ($n = 798$), all flies from experiment 1 and a subset of flies from experiment 2 (Table 1, groups 1 and 2) were further analyzed for *ts* genotype ($n = 438$). Differences in mean blood uptake as a result of treatments that were observed in the parent group (analysis of variance [ANOVA]: $F = 6.957$, $P = 0.001$, $n = 798$) were detected similarly in this subset (ANOVA: $F = 5.108$, $P = 0.006$, $n = 438$). Additionally, blood uptake of specific treatment groups did not differ between the two vaccine trials (Table 3; ANOVA: $F = 0.100$, $P = 0.785$, $n = 26$), allowing the two data sets to be combined for testing isoform effects.

Table 3. Blood uptake by flies feeding on experimental calves

Vaccine	Set of all flies mean ± SEM (n)	Genotype subset mean ± SEM (n)
OVA (control)	0.95 ± 0.04 (304)	0.90 ± 0.05 (178)
TB8	0.79 ± 0.04 (275)	0.71 ± 0.05 (144)
TS9	0.73 ± 0.05 (219)	0.73 ± 0.06 (116)

Blood uptake was decreased for flies fed on TB8- or TS9-immunized cattle for the set of all flies (ANOVA: $F = 6.957$, $P = 0.001$, $n = 798$) and for the genotype subset (ANOVA: $F = 5.108$, $P = 0.006$, $n = 438$). Mean blood volumes for treatment groups were not different between the set of all flies and the genotype subset (ANOVA: $F = 0.100$, $P = 0.755$, $n = 26$).

The increased uptake of blood by horn flies feeding on control cattle after sensitization by prior horn fly biting, as reported earlier (Cupp et al. 2004), was confirmed in the second trial (Fig. 2; ANOVA: $F = 5.230$, $P = 0.023$, $n = 642$). When cattle were vaccinated with rTB8 or rTS9, however, no increase in blood uptake occurred (Fig. 2).

Further analysis of blood uptake by specific genotype revealed a differential effect of *ts* alleles on feeding success. Ninety-nine percent of all TS genes within the experimental horn fly population were composed of five *ts* alleles, *ts2*, *ts8*, *ts9*, *ts10*, and *tb8*, with *ts9* and *ts10* accounting for 67% of the total (Fig. 3). Although the mean blood uptake of the total population of flies was decreased when flies fed on cattle vaccinated with rTB8, the effect was because of an impact only on flies with *ts8*, *ts9*, and *tb8* alleles (Fig. 4), which together

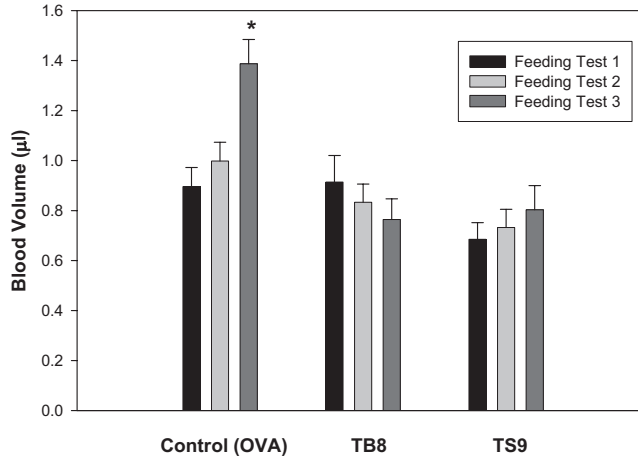

Fig. 2. Blood uptake (mean ± SEM) by horn flies fed on control- or TS-vaccinated cattle in three sequential feeding tests.

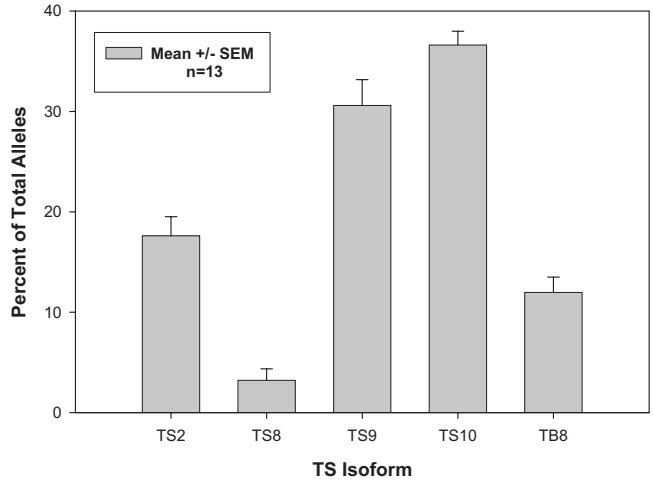

Fig. 3. Distribution of the five most common *ts* alleles (99%) of experimental flies used in feeding trials (mean ± SEM; $n = 13$ fly groups).

comprised 46% of the population (Fig. 3). In addition to flies carrying *ts9* and *tb8* alleles, flies with *ts2* alleles (17%), but not those with *ts8*, were inhibited in feeding from cattle when rTS9 was used as the vaccination protein (Fig. 4). In contrast, flies carrying *ts10* alleles (36%) were as successful in feeding from TB8- or TS9-vaccinated calves as from controls (Fig. 4).

Data in Tables 4–9 show blood uptake when flies were homozygous or heterozygous for specific *ts* gene alleles. Blood volumes obtained from TS-vaccinated calves were similar for both homozygous and heterozygous flies carrying alleles *ts2* (Table 4), *ts9* (Table 5), and *tb8* (Table 6). Sorting of feeding data by number of *ts8* alleles (Table 7) emphasized the very low frequency of this genotype in the colony horn fly population used for experimental studies. ANOVA, however, detected an increase in blood uptake for *ts8* homozygous flies compared with flies with a single *ts8*

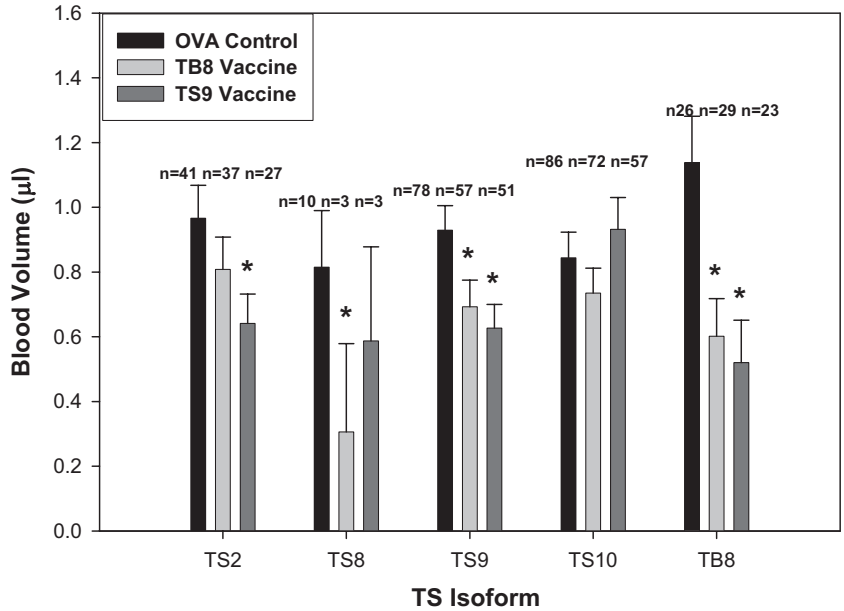

Fig. 4. Relationship of blood uptake to *ts* genotype of horn flies when fed on control-, rTB8-, or rTS9-vaccinated cattle. Asterisk (*) indicates a significant effect of fly genotype on blood feeding from TS-vaccinated cattle compared with control cattle. ANOVA hypothesis test: *ts2* genotype, vaccine TS9: $F = 5.571$, df = 1, $P = 0.020$, $n = 68$; *ts8* genotype, vaccine TB8: $F = 13.993$, df = 1, $P = 0.005$, $n = 13$; *ts9* genotype, vaccine TB8: $F = 4.568$, df = 1, $P = 0.034$, $n = 135$; vaccine TS9: $F = 6.851$, df = 1, $P = 0.010$, $n = 129$; *tb8* genotype, vaccine TB8: $F = 4.828$, df = 1, $P = 0.031$, $n = 55$; vaccine TS9: $F = 10.598$, df = 1, $P = 0.002$, $n = 49$.

Table 4. Flies with *ts2* alleles

	Vaccine: OVA (control)	Vaccine: TB8	Vaccine: TS9
1 allele	$n = 26$	$n = 25$	$n = 12$
	0.983 ± 0.130	0.847 ± 0.122	0.578 ± 0.112
2 alleles	$n = 15$	$n = 12$	$n = 15$
	0.935 ± 0.171	0.727 ± 0.180	0.691 ± 0.139

Blood uptake did not differ between heterozygous and homozygous *ts2* flies (ANOVA: $F = 0.060$, $P = 0.807$, $n = 105$).

Table 6. Flies with *tb8* alleles

	Vaccine: OVA (control)	Vaccine: TB8	Vaccine: TS9
1 allele	$n = 19$	$n = 19$	$n = 18$
	1.257 ± 0.173	0.609 ± 0.142	0.546 ± 0.164
2 alleles	$n = 7$	$n = 10$	$n = 5$
	0.816 ± 0.221	0.589 ± 0.211	0.427 ± 0.152

Blood uptake did not differ between heterozygous and homozygous *tb8* flies (ANOVA: $F = 1.678$, $P = 0.199$, $n = 78$).

allele (ANOVA: $F = 19.314$, $P = 0.002$, $n = 16$). A similar analysis of horn flies with *ts10* alleles uncovered a significant decrease in blood uptake from control-vaccinated cattle when both genes were *ts10* (Table 8; ANOVA: $F = 4.190$, $P = 0.044$, $n = 86$). Feeding of *ts10* flies on control-vaccinated calves was explored further by sorting data by feeding times. This analysis revealed that lower blood uptake of *ts10* homozygous flies from control cattle occurred only for the first two feedings. However, after extensive field exposure of cattle to horn flies that occurred after the second feeding test, blood meal volumes of *ts10* genotype flies reached that of other genotypes in the third feeding test (Table 9).

Discussion

The presence of a prominent blood clot-inhibiting protein in the saliva of both male and female horn flies implied a relevant, and perhaps essential, action in their blood-feeding life style (Cupp et al. 1998). Experimental feeding studies supported the importance of salivary factors when blood meal volumes of horn flies increased if their hosts, NZW rabbits or cattle, had been sensitized to saliva by previous horn fly feeding (Cupp et al. 2004, 2009). That effect of host sensitization on blood uptake volumes was confirmed in the second cattle vaccine experiment described in this study. Inhibition of the phenomenon by vaccination with two different rTS proteins strongly linked the feeding response to the TS component of horn fly saliva.

Because of the polymorphic nature of the *ts* gene, further analysis tested a potential relationship of feeding to each of the five most prominent *ts* alleles within the horn fly genome. A correlation was observed for horn flies fed on control NZW rabbits, wherein differences in blood uptake among flies within a feeding group were linked to specific *ts* genotype (Cupp et al. 2009). In those studies, flies possessing one or more *ts10* alleles obtained larger blood volumes after 20 min of feeding than did flies with one or more *ts9* alleles. Although five major *ts* alleles have been identified in horn flies, those two alleles comprised 68% of the *ts* genes in the Kerrville, Texas laboratory strain of horn flies that was used for experimental studies.

The reversal in outcomes for *ts9* and *ts10* genotype flies fed on cattle compared with rabbits was striking. In contrast to their superior feeding on a laboratory host, NZW rabbits, homozygous *ts10* horn flies were less successful than the *ts9* genotype in initial feedings on a natural host, dairy cattle. These differential effects highlight the potential importance of host-specific interactions with salivary TS in facilitating blood feeding of horn flies.

Sustained feeding of *ts10* genotype flies tested on rTS9-immunized cattle, in contrast to the inhibited feeding of flies carrying *ts2*, *ts9*, and *tb8* alleles, confirms a difference in cattle immune recognition of the TS10 protein isoform. This putative escape of *ts10* genotype flies from an immune response to TS9 protein may account for the continued prevalence of this genotype in field populations, in spite of their suboptimal feeding on cattle. Alleles for *ts10* constituted 17% of the total *ts* genes within a collection of horn flies from dairy cattle in Alabama (Zhang et al. 2001) and 20% of *ts10* alleles in flies collected from Camp Stanley, Texas (Untalan et al. 2006), compared with *ts9* alleles of 42 and 28%, respectively. Distribution of *ts* genotype horn flies did not appear to be uniform among cattle, however, when density on Texas field cattle was taken into account. Analysis of flies collected from cattle designated as low carriers found that the number of flies carrying one or more *ts10* alleles exceeded those with one or more *ts9* alleles (25 flies with *ts10* compared with 19 with *ts9*). The opposite relationship occurred on cattle designated as high carriers of horn flies in which 14 of 46 flies carried *ts10* alleles (30%) compared with 25 with *ts9* (65%; Untalan et al. 2006).

Table 5. Flies with *ts9* alleles

	Vaccine: OVA (control)	Vaccine: TB8	Vaccine: TS9
1 allele	$n = 39$	$n = 27$	$n = 33$
	0.959 ± 0.110	0.623 ± 0.120	0.678 ± 0.095
2 alleles	$n = 39$	$n = 30$	$n = 18$
	0.898 ± 0.105	0.757 ± 0.114	0.533 ± 0.114

Blood uptake did not differ between heterozygous and homozygous *ts9* flies (ANOVA: $F = 0.020$, $P = 0.888$, $n = 186$).

Table 7. Flies with *ts8* alleles

	Vaccine: OVA (control)	Vaccine: TB8	Vaccine: TS9
1 allele	$n = 3$	$n = 0$	$n = 1$
	0.331 ± 0.210		0.007
2 alleles	$n = 7$	$n = 3$	$n = 2$
	1.023 ± 0.189	0.306 ± 0.273	0.877 ± 0.035

Blood uptake was greater for *ts8* homozygous flies, $P = 0.002$ (ANOVA: $F = 19.314$, $P = 0.002$, $n = 16$).

Table 8. Flies with *ts10* alleles

	Vaccine: OVA (control)	Vaccine: TB8	Vaccine: TS9
1 allele	$n = 44$	$n = 41$	$n = 34$
	0.998 ± 0.099	0.765 ± 0.113	0.975 ± 0.137
2 alleles	$n = 42$	$n = 31$	$n = 23$
	0.682 ± 0.120	0.694 ± 0.099	0.869 ± 0.136

Blood uptake was lower for homozygous *ts10* flies fed on control cattle (ANOVA: $F = 4.190$, $P = 0.044$, $n = 86$).

Table 9. Flies with *ts10* alleles fed on control cattle

	Feed time 1	Feed time 2	Feed time 3
1 allele	$n = 10$	$n = 25$	$n = 9$
	0.864 ± 0.202	0.920 ± 0.238	1.365 ± 0.156
2 alleles	$n = 17$	$n = 17$	$n = 8$
	0.409 ± 0.151	0.549 ± 0.103	1.550 ± 0.376

The concept of a particular horn fly infestation number on cattle that reaches an economic threshold is widely accepted (Steelman 1976, Pruett et al. 2003, Jensen et al. 2004) and often is used to guide current methods of treatment. Cattle that carried high numbers of horn flies in 1 yr were found to retain that status in the following year, and the high levels of infestation were retained when individuals were moved to different herds (Jensen et al. 2004). Selection of cattle for resistance has been suggested as one means for keeping horn fly populations below the economic threshold (Pruett et al. 2003). In areas where *ts9* genotype horn flies predominate in populations on high carrier cattle, as described in the Texas study (Untalan et al. 2006), vaccination with rTS9 should provide a reasonably simple and effective method of reducing fly numbers by its impact on essential nutrition of the majority genotype.

Horn flies of *ts2* genotype do not occur in high numbers among field-collected flies (Zhang et al. 2001, Untalan et al. 2006), but are more common in the Texas colony flies used for these feeding studies. The structure in the mature thrombin-inhibiting TS2 peptide varies from TS9 only at position 10, where an asparagine (N) replaces an aspartic acid (D) (Fig. 5). Both surface probability and antigenic index are predicted to be lower for TS2 peptide, with the region affected extending from the leucine (L) at position 7 through the aspartic acid (D) at position 12. Although vaccination with rTS9 disrupted normal blood feeding by *ts2* genotype flies, immunization with rTB8 was ineffective. This difference suggests that the substitution of an N for D at position 10 does have significant effects on surface properties that mask an important epitope recognized by immune response to rTB8 vaccination. In addition to the three amino acid differences in the active thrombin-inhibiting peptides, rTB8 vaccine varied from rTS9 vaccine in the composition and location of the expression tags used for vaccine production (Fig. 1), which has the potential to affect surface properties and immune response.

In contrast to *ts2*, horn flies of *ts8* genotype are prevalent within many field populations, but occur in relatively low numbers within the Texas colony flies used for laboratory studies. Horn flies collected from dairy cattle in Alabama (mixed breed, primarily Holstein, *Bos taurus*) had *ts8* alleles equal to *ts10* (Zhang et al. 2001). Additionally, in three separate physical analyses of purified TS in saliva pools collected from horn flies obtained from the same herd, the quantity of peptide with molecular mass equal to TS8 was greater than that of TS10 and was exceeded only by peptide with mass equal to TS9 (M.S.C., unpublished observation; for one analysis, see Fig. 6, Zhang et al. 2002). The *ts8* allele was well represented in field-collected horn flies of Texas. In one location (Camp Stanley, Texas), the *ts8* allele made up 37.5% of the population and, in other collections, was a common allele among horn flies collected from low-carrier (24.5%) and high-carrier bulls (28.0%) (Untalan et al. 2006). Thus, an understanding of the feeding response of horn flies with *ts8* genotype is of interest and is likely to be important in developing effective horn fly control that is based on targeting salivary proteins, especially in areas of *ts8* genotype predominance.

The low frequency of the *ts8* genotype within the Texas colony horn flies and the lack of external markers for selecting specific genotypes limited the strength of conclusions about TS8 that could be drawn from these studies. Among the 438 flies (876 alleles), only 16 flies carried one or more *ts8* alleles ($n = 28$ alleles). Although the low numbers of observation in most groups warrant caution, ANOVA detected higher blood uptake for homozygous *ts8* flies than for those with only one *ts8* allele. All heterozygous *ts8* flies in these studies also carried *ts9* alleles and would be expected to have sensitivity to TB8 or TS9 vaccination.

```
      1                                                  50
TS-2  SAGPITLQLN DDDDDDSGIP IFEMDDEDED SNDNQKFPLS FERFPENEKN
TS-8  SAGPITLQLD DDDDDDSGIP IFEMDDEDED SNDNQKFPLS FERFPENEKN
TS-9  SAGPITLQLD DDDDDDSGIP IFEMDDEDED SNDNQKFPLS FERFPENEKN
TS-10 SAGPITLQLD DDDDDDSGIP IFEMDDEDED SNDNQKFPLS FERFPENEKN
TB-8  SAGPITLQLD DDDDDDSGIP IFEMDDEDVD SNDNQKFPLS FERFPENEKN

      51                                 81
TS-2  QEGLRARFNK FMAKFTSLFG RRRGVDVPNA A
TS-8  QEGLRARFNK FMAKFTSLFG RRRSVDVPNA A
TS-9  QEGLRARFNK FMAKFTSLFG RRRGVDVPNA A
TS-10 QVGLRARFNK FMAKFTSLFG RRRGVNVPNA A
TB-8  QVGLRARFNK FMAKFTSLFG RRRGVNVPNA A
```

Fig. 5. Amino acid composition of the five major isoforms of TS produced in saliva of horn flies collected from cattle in Alabama and Texas, and from horn flies within a Texas colony. Amino acid variants are highlighted with bold lettering.

Further studies of *ts* genes in field-collected horn flies from a number of sites within the United States, South America, and Europe support the importance of TS8 peptide in horn fly saliva and will be presented in a forthcoming report (D. Z., M.S.C., K. Moulton, S. Coscaron, and E.W.C.; unpublished observation).

In summary, these studies with a natural host (cattle), in addition to previous studies with a laboratory model host (rabbit), demonstrated a dynamic interaction between TS protein isoforms in horn fly saliva and a fly's ability to blood feed. Differences in response to TS isoforms between rabbit and cattle hosts indicate the importance of specific host factors to genetic selection of horn flies, including salivary TS. Immunization of Alabama dairy cattle with a recombinant form of the TS isoform most prevalent in local horn fly populations (rTS9) resulted in a significant decrease in blood uptake by flies carrying three of the five major *ts* alleles, *ts2*, *ts9*, and *tb8*. The lack of a significant impact of rTS9 vaccination on feeding of flies with *ts10* or homozygous *ts8* alleles indicates that a vaccine mixture that includes rTS9 and one or both of those isoforms would be most successful for widespread control of this economically important ectoparasite.

Acknowledgments

The important assistance of Eugene Blair of the Auburn University College of Veterinary Medicine in carrying out cattle experiments and Mathew Waldon of the Kerrville Laboratory in providing timely shipments of fly pupae to accommodate the experimental schedule is gratefully acknowledged. Horn fly pupae were provided by the United States Department of Agriculture Livestock Insects Research Laboratory (Kerrville, TX). This work was supported by the National Research Initiative of the United States Department of Agriculture Cooperative State Research, Education and Extension Service, Grant 2001-35302-1092. We gratefully acknowledge financial support provided by Novartis Animal Health, the Auburn University Department of Entomology and Plant Pathology, and the Auburn University Colleges of Agriculture and Veterinary Medicine.

References Cited

Briegel, H., A. O. Lea, and M. J. Klowden. 1979. Hemoglobinometry as a method for measuring blood meal sizes of mosquitoes. J. Med. Entomol. 15: 235–238.

Cupp, E. W., M. S. Cupp, J.M.C. Ribeiro, and S. E. Kunz. 1998. Blood-feeding strategy of *Haematobia irritans* (Diptera: Muscidae). J. Med. Entomol. 35: 591–595.

Cupp, M. S., D. Zhang, and E. W. Cupp. 2000. Horn fly saliva targets thrombin action in hemostasis. J. Med. Entomol. 37: 416–421.

Cupp, M. S., E. W. Cupp, C. Navarre, N. Wisnewski, K. S. Brandt, G. M. Silver, D. Zhang, and V. Panangala. 2004. Evaluation of a recombinant salivary gland protein (thrombostasin) as a vaccine candidate to disrupt blood-feeding by horn flies. Vaccine 22: 2285–2297.

Cupp, M. S., E. W. Cupp, D. Zhang, X. Yue, L. Todd, V. Panangala, C. Navarre, and E. Whitley. 2009. Salivary gland thrombostasin isoforms differentially regulate blood uptake of horn flies fed on New Zealand White rabbits. J. Med. Entomol. 46: 351–357.

Derouen, S. M., J. E. Miller, L. D. Foil, and G. T. Gentry. 2009. Control of horn flies (*Haematobia irritans*) and gastrointestinal parasites and its relation with cow-calf performance. Vet. Parasitol. 162: 320–326.

Guglielmone, A. A., E. Gimeno, J. Idiart, W. F. Fisher, M. M. Volpogni, O. Quaino, O. S. Anziani, S. G. Flores, and O. Warnke. 1999. Skin lesions and cattle hide damage from *Haematobia irritans* infestations. Med. Vet. Entomol. 13: 324–329.

Hibler, C. P. 1966. Development of *Stephanofilaria stilesi* in the horn fly. J. Parasitol. 52: 890–898.

Jensen, K.-M.V., J. B. Jespersen, M. A. Birkett, J. A. Pickett, G. Thomas, L. J. Wadhams, and C. M. Woodcock. 2004. Variation in the load of the horn fly, *Haematobia irritans*, in cattle herds is determined by the presence or absence of individual heifers. Med. Vet. Entomol. 18: 275–280.

Owens, W. E., S. P. Oliver, B. E. Gillespie, C. H. Ray, and S. C. Nickerson. 1998. Role of horn flies (*Haematobia irritans*) in *Staphylococcus aureus*-induced mastitis in dairy heifers. Am. J. Vet. Res. 59: 1122–1124.

Owens, W. E., S. C. Nickerson, and C. H. Ray. 2002. Effect of a pour-on and fly tag insecticide combination in controlling horn flies and *Staphylococcus aureus* mastitis in dairy heifers. 2002. J. Anim. Vet. Adv. 1: 200–201.

Oyarzún, M. P., A. Quiroz, and M. A. Birkett. 2008. Insecticide resistance in the horn fly: alternative control strategies. Med. Vet. Entomol. 22: 188–202.

Pruett, J. H., C. D. Steelman, J. A. Miller, J. M. Pound, and J. E. George. 2003. Distribution of horn flies on individual cows as a percentage of the total horn fly population. Vet. Parasitol. 116: 251–258.

Sanson, D. W., A. A. DeRosa, G. R. Oremus, and L. D. Foil. 2003. Effect of horn fly and internal parasite control on growth of beef heifers. Vet. Parasitol. 117: 291–300.

Steelman, C. D. 1976. Effects of external and internal arthropod parasites on domestic livestock production. Annu. Rev. Entomol. 21: 155–178.

Untalan, P. M., J. H. Pruett, H. N. Atteberry, and C. D. Steelman. 2006. Thrombostasin isoform frequency in a central Texas field population of the horn fly, *Haematobia irritans*. Vet. Parasitol. 142: 359–366.

Zhang, D., M. S. Cupp, and E. W. Cupp. 2001. Polymorphism of thrombostasin revealed in cDNA library and genomic DNA. Mol. Genet. Genomics 266: 296–302.

Zhang, D., M. S. Cupp, and E. W. Cupp. 2002. Thrombostasin: purification, molecular cloning, and expression of a novel antithrombin protein from horn fly saliva. Insect Biochem. Mol. Biol. 32: 321–330.

Received 13 October 2009; accepted 3 February 2010.