Fingerprint Profiles of Cuticular Fatty Acids in Three Adult *Rhipicephalus* Tick species: A New Tool for Identification

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ABSTRACT

Ticks are obligatory blood parasites responsible for transmitting a variety of diseases to humans and animals. Since ticks share similar morphological features, accurate identification of tick species is important for control and management of disease-associated risks. Here, we report the application of gas chromatography mass spectrometry for analyses of cuticular fatty acid profiles for specific identification of female *Rhipicephalus annulatus, Rhipicephalus bursa* and *Rhipicephalus sanguineus* and establish the reliance of this assay on their nutritional status. Our findings demonstrated a unique pattern for each species under fed and unfed conditions. The cuticular fatty acid fractions were composed of a mixture of saturated and unsaturated straight-chain, terminally methylated and hydroxylated fatty acids. Our results show significant differences between the species tested under identical nutritional status, whereas the cuticular fatty acid profile pattern under fed versus unfed conditions in each species were similar. *R. annulatus* was distinguished by the high abundance of C10 to C14 fatty acids and the presence of 2-OH-C16, in contrast to *R. sanguineus* and *R. bursa* forming a higher percentage of C16 to C18. In addition, *R. bursa* was clearly distinguished from *R. sanguineus* by its high intensity of C14 and C20 as well as the presence of C12. Based on these results, we have established the ratio of the relative average abundance values of C16 to C14 as a Species Differentiation Index, enabling us to identify each of these species independent of its nutritional status. We suggest that fatty acid profiling may be useful as a relatively simple and reliable method to determine the species-specific identity of ticks.

Keywords: Rhipicephalus ticks, fatty acid profile, mass spectrometry.

INTRODUCTION

Ticks, of which there are more than 900 species world-wide, are parasitic arthropods that mostly feed on the blood of mammals, while other feed on the blood of birds, reptiles and even amphibians. Ticks are regarded as the most important vectors of pathogens transmitting diseases in the temperate zone (1). Tick-borne diseases are of significant importance to human and animal health, as they cause high morbidity and may be fatal; onset of fever and rash in humans following a tick bite signify a medical emergency (1, 2, 3). As the incidence of tick-borne illnesses increases and the geographic areas in which they are found expand, it becomes increasingly important to distinguish between the highly diverse, and often overlapping clinical presentations of diseases (4). In the Mediterranean region, *Rhipicephalus sanguineus* is a common transmitter of *Rickettsia conorii,* the causative agent of Mediterranean Spotted Fever, also called Tick Bite Fever and Boutonneuse Fever (5). *Rhipicephalus annulatus* is particularly important in transmitting babesiosis, which is caused by *Babesia bigemina* and *Babesia bovis,* as well as anaplas-

mosis. *Rhipicephalus bursa* transmits *Babesia*, *Theileria*, and *Anaplasma* spp. to livestock (3). Accurate tick species identification is very important in the early stage recognition and management of disease-risk.

At present, the techniques applied for diagnostic and taxonomic tick identification rely mostly on morphological differences and to a lesser extent on molecular markers. Species specific enzymatic polymorphisms, as well as nuclear and mitochondrial rRNA sequences have been reported (6, 7, 8). Recent applications of molecular techniques such as PCR for the identification of ticks are rarely used in clinical diagnostic settings, mainly due to the lack of species-specific tick DNA markers. As most diagnostic work is still based on differences in morphological features, which requires highly trained and experienced personal and can be time consuming, a fast and easy assay is warranted for unique tick species identification.

Total fatty acid profiles are increasingly used as a chemotaxonomic tool for the identification and classification of bacteria and fungi for more than two decades (9, 10, 11, 12, 13). The composition and proportion of total cellular fatty acids can be used to characterize a species, the developmental stage and the physiological state of the microorganism. The fatty acid profile analysis is accomplished by converting whole cell fatty acids into their corresponding methyl esters and identifying each component between 9 and 24 carbons in length by gas chromatography mass spectrometry (12).

Since the outer waxy cuticular layer of *Ixodid* ticks is composed of a mixture of various lipid classes, such as free and esterified sterols, esters of long chain fatty acids and alcohols, and triglycerols (14), it can potentially be used to establish a species specific fatty acid profile, which might be further utilized as a chemotaxonomic tool for tick identification.

The aim of the current study was to characterize the total fatty acid profile between C10 and C24 carbons in length of three *Rhipicephalus* species, *R. annulatus, R. sanguineus* and *R. bursa* under fed (engorged) and unfed conditions, and to determine the utility of the cuticular fatty acid composition as a potential chemotaxonomic tool for species specific identification.

MATERIALS AND METHODS

Reagents and materials

Dichloromethane, hexane, methanol, methyl tert-butyl ether, hydrochloric acid 37% Bacterial Acid Methyl Ester (BAME Mix, Supelco, Sigma Aldrich, Jerusalem, Israel) mix and sodium hydroxide pellets were purchased from Sigma (Sigma Aldrich, Jerusalem, Israel). All glassware used was rinsed once with redistilled dichloromethane.

Ticks

Adult engorged and unfed females of *Rhipicephalus annulatus, Rhipicephalus bursa* and *Rhipicephalus sanguineus* were laboratory reared at the Kimron Veterinary Institute, Israel. Thirty ticks from each species and of each nutritional status were collected. *R. sanguineus* and *R. bursa* ticks were collected from domestic infested rabbits, while *R. annulatus* ticks were collected from domestic cattle. Ticks from each species were placed separately in Petri dishes for 24 hours before being processed for cuticular fatty acid analyses.

Extraction procedure and preparation of fatty acid methyl esters

Freshly distilled dichloromethane (2.0 mL) was poured into 10 mL glass vials each containing 2 engorged ticks of the same species, or 10 unfed ticks of the same species. The ticks in the glass vials were sonicated for 5 min at 43 kHz, after which the dichloromethane was transferred into a new glass tube and the preparations evaporated to dryness at 25°C under a stream of N_2.

Methyl esterification of total cuticular fatty acids was accomplished according to the procedure published by Sasser *et al.* (12). Briefly, 1 ml of 4 M methanolic/NaOH solution was added to each tube containing dried cuticular extract and heated in a water bath at 100°C for 30 min. The methylation of hydrolyzed fatty acids was accomplished by adding 2.0 ml of 3 M methanolic/HCl solution to the cooled tubes followed by heating at 80°C for 10 min. Fatty acid methyl esters were extracted with 1.25 ml of a hexane: methyl tert-butyl ether (1:1) mix and subsequently washed with 0.3 M NaOH solution. The organic phase was transferred directly into a glass vial and injected into the gas chromatography / mass spectrometry (GC/MS) instrument (Agilent 7890A Model equipped with an Agilent 5975C VL MSD, Santa Clara, USA).

Analysis of total cuticular fatty acid methyl esters

The total cuticular fatty acid methyl esters were analyzed by GC/MS. The separation of fatty acid methyl esters

was achieved using DB-5MS capillary column (30 meter length, 0.25 mm internal diameter, 0.25 μm film thickness). The temperature program was as follows: injector temperature, 230 °C; initial temperature, 180 °C for 3 min; gradient of 17 °C/min until 250 °C; gradient of 10 °C until 300 °C; hold time, 2 min. The mass spectrometer parameters were set as follows: source temperature, 230 °C; transfer line, 230 °C; positive ion monitoring; Electron impact 70 eV. Qualitative analysis was performed by comparing the pure mass spectrum of each eluting compound with those in the National Institute of Standards and Technology (NIST-05) mass spectral library. In addition, the identity of each eluting fatty acid methyl ester was verified by comparing the mass spectrum and retention time with a corresponding standard mixture composed of 26 known fatty acid methyl esters (BAME Mix, Supelco, Sigma Aldrich, Israel). Integration of the total ion chromatogram was performed using Agilent Chemstation data analysis software (Agilent Technologies®, Santa Clara, USA). Peak areas were converted to percentages of the total fatty acid fraction, excluding peaks with an area fraction below 1%. This procedure standardized runs by eliminating differences in sample volumes related to the size of tick specimens (15, 16).

Statistics

Mean percentage abundance of total peak area was compared between the three tick species as well as within the same species under different nutritional conditions (Table 1), by utilizing a multiple comparison ANOVA test and a student t-test with a p value of 0.05, respectively.

The statistical test was performed with GraphPad Prism version 5.04 for Windows (GraphPad Software, La Jolla California USA).

RESULTS

The analysis of cuticular fatty acid composition of the three *Rhipicephalus* tick species by GC/MS technique revealed the presence of a mixture of saturated, unsaturated and mono hydroxylated fatty acids with a chain length between C10 to C24 carbons (Table 1, Figure 1). The composition and type of cuticular fatty acids of engorged and unfed ticks within each species were identical (Table 1, Figure 1). On the other hand, the relative abundance of several fatty acids within each species under fed and unfed conditions revealed significant differences (Table 1).

The transition of *R. annulatus* from unfed to engorged nutritional status significantly increased the relative abundance of all saturated long-chain fatty acids with C18 to C24 carbon chain number, whereas, the relatively polar hydroxylated fatty acid, 2-OH-palmitic acid, had a 3-fold lower relative abundance in engorged females (Table 1). Engorged *R. bursa*, on the other hand, displayed a decrease in the relative abundance of stearic acid (C18), but demonstrated a 3-fold higher abundance of arachidic acid (C20) as compared with its unfed state (Table 1). The nutritional status of *R. sanguineus* affected significantly only the relative abundance value of C20, which was 2.5-fold higher in engorged females. From the data depicted in Table 1, a general trend can be observed, in which the relative abundance of some saturated long-chain fatty acids above C18 is increased following the transition from unfed to fed state.

Under both nutritional conditions, the fatty acids capric acid (C10) to myristic acid (C14) constitute more than 50% of the total cuticular fatty acids of *R. annulatus*, whereas the longer chain fatty acids palmitic (C16) to lignoceric acid (C24) constitute more than 90% of the total cuticular fatty acid profile of *R. sanguineus* and *R. bursa* (Table 1). *R. annulatus* revealed the most complex fatty acid profile, composed of a versatile mixture of 13 fatty acids from C10 to C24, including 2 mono hydroxylated (2-OH-C14; 2-OH-C16), 2 unsaturated (linoleic and oleic acid), 1 branched (13-methyl-C14) and 8 saturated straight chain fatty acids, occurring as a continuous homolog series from C10 to C24. In addition, only *R. annulatus* displayed two unique fatty acids among the tested species, namely 2-OH-C14 (myristic acid) and 2-OH-C16 (palmitic acid) (Table 1).

R. sanguineus displayed the lowest number of cuticular fatty acids, among which there were 4 saturated straight chain fatty acids (C14, C16, C18 and C20), 2 unsaturated fatty acids (cis-9-C18:1 and cis, cis-9, 12-C18:2), and 1 terminally branched mono-methyl fatty acid (13-methyl-myristic acid). The most prominent peaks with a relative abundance values above 35% were C16 and C18 (Table 1). *R. bursa* exhibited a similar fatty acid composition to *R. sanguineus*, with the exception of C12, C22 and C24 possessing abundance values below 5% (Table 1).

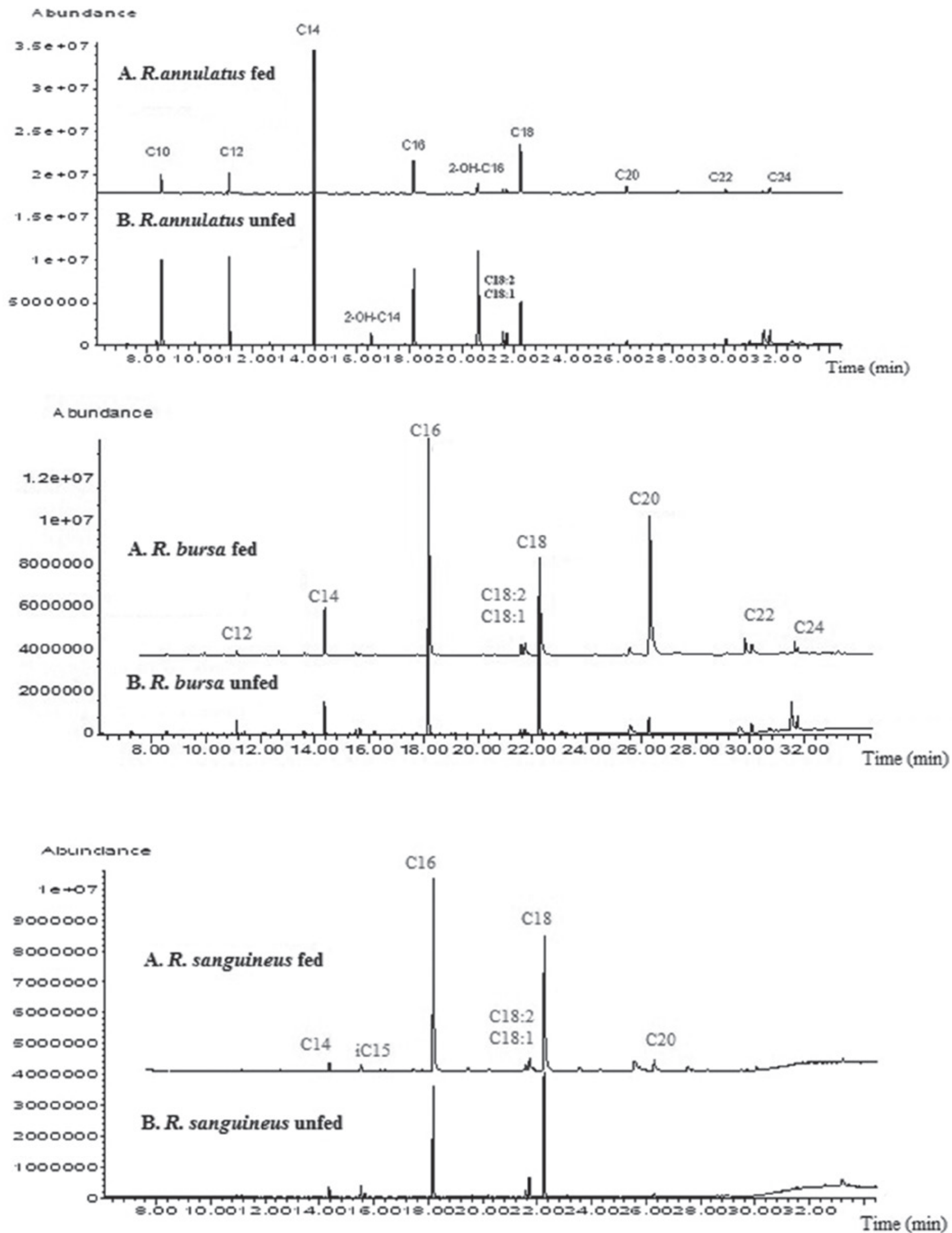

Figure 1: Total ion chromatograms of cuticular fatty acid profile for engorged and unfed adult female *Rhipicephalus bursa, R. sanguineus* and *R. annulatus*. Time axis is given in min. Each peak in the chromatogram is designated with its corresponding fatty acid: C10, capric acid; C12, lauric acid; C14, myristic acid; 2-OH-C14, iC15, 13-methyl-myristic acid; 2-hydroxy-myristic acid; C16, palmitic acid; 2-OH-C16, 2-hydroxy-palmitic acid; cis, cis-9, 12-C18:2, linoleic acid; cis-9-C18:1, oleic acid; C18, stearic acid; C20, arachidic acid; C22, behenic acid; C24, lignoceric acid.

Table 1: Percentage of total cuticular fatty acids extracted from three adult *Rhipicephalus* species under fed and unfed conditions.

Fatty Acid[a]	RT (min)[b]	R. Bursa		R. sanguineus		R. annulatus	
		Fed Mean % (n=30)[c] ± SD[d]	Unfed Mean % (n=30) ± SD	Fed Mean % (n=30) ± SD	Unfed Mean % (n=30) ± SD	Fed Mean % (n=30) ± SD	Unfed Mean % (n=30) ± SD
C10 (capric acid)	8.55	ND[e]	ND	ND	ND	7.9 ± 1.6	11.6 ± 2.6
C12 (lauric acid)**	11.14	1.5 ± 0.5	2.3 ± 0.8	ND	ND	7.0 ± 1.5	10.1 ± 2.7
C14 (myristic acid)**	14.36	5.8 ± 0.4	6.5 ± 1.8	1.5 ± 0.3	1.7 ± 0.8	34.6 ± 3.3	37.6 ± 4.0
iC15 (13-methyl-myristic acid)	15.51	1.0>	1.0>	3.6 ± 0.7	3.5 ± 1.0	ND	ND
2-OH-C14	16.54	ND	ND	ND	ND	1.0>	1.0>
C16 (palmitic acid)**	18.19	31.3 ± 2.6	33.2 ± 2.1	42.9 ± 5.2	40.1 ± 3.2	12.6 ± 1.5	12.1 ± 1.9
2-OH-C16	20.62	ND	ND	ND	ND	5.5 ± 1.3*	16.7 ± 2.5
cis, cis-9, 12-C18:2 (linoleic acid)	21.59	1.8 ± 0.4	1.4 ± 0.2	1.9 ± 0.4	2.4 ± 0.6	1.4 ± 0.6	1.2 ± 0.3
cis-9-C18:1 (oleic acid)	21.73	2.0 ± 0.7	1.6 ± 0.5	2.6 ± 0.8	3.4 ± 0.9	1.6 ± 0.3	1.0 ± 0.1
C18 (stearic acid)	22.27	20.7 ± 3.3*	36.4 ± 3.5	41.5 ± 4.8	44.7 ± 4.5	20.9 ± 2.5*	6.8 ± 1.7
C20 (arachidic acid)	26.31	31.1 ± 2.0*	5.0 ± 1.1	3.3 ± 0.3*	2.5 ± 0.1	3.8 ± 1.1*	1>
C22 (behenic acid)	30.06	2.4 ± 0.3	2.2 ± 0.4	ND	ND	2.1 ± 0.9*	1>
C24 (lignoceric acid)	31.75	2.9 ± 0.9	3.4 ± 0.8	ND	ND	2.2 ± 0.6*	1>

* Average relative abundance values within the same species under fed vs. unfed conditions were significantly ($p < 0.05$) different. The mean abundance values of the same species under different nutritional status were analyzed using student t test with a P-value of 0.05 considered to be significant.

** Average relative abundance values between the three species under similar nutritional status were significantly different ($p < 0.05$). The mean abundance values were analyzed using a multiple comparison ANOVA test with a p-value of < 0.05 considered to be significant.

[a] Fatty acids that co-elute as their corresponding methyl esters listed in order of their retention time.

[b] RT= retention time in minutes.

[c] mean (n) = mean percentage contribution to total area of all peaks with the total number of ticks in parenthesis.

[d] SD = standard deviation.

[e] ND = not detected.

DISCUSSION

The usefulness of fatty acid profile analysis in taxonomy has been established previously for bacteria and fungi (9, 11, 12). This study demonstrates the usefulness of cuticular fatty acid composition as a valuable tool for the identification of *Rhipicephalus* species under fed and unfed conditions. The importance of correct identification of Ixodid ticks, when morphological features are obscured (in immature stages, or when the female is engorged), led us to focus on the development of a rapid and reliable technique to identify engorged adult female ticks. Here we demonstrated the feasibility of this method by characterizing three closely related *Rhipicephalus* species based on their fatty acid profile.

The fatty acid profile for the three *Rhipicephalus* species displayed consistent and species specific differences in the relative abundance of several fatty acids, enabling easy identification of these species. The unique cuticular fatty acid composition of each species under fed and unfed conditions (Table 1) enabled a clear distinction between *R. annulatus, R. sanguineus* and *R. bursa.* Based on the obtained results presented in Table 1, we have found that the ratio of C16 to C14 under both nutritional conditions yielded a highly reproducible and species-specific value, which could be used as a species differentiation index (Table 2). The ratio of C16 to C14 was preferentially chosen over other fatty acid ratios, since the following favorable conditions were met for both fatty acids: 1) C16 and C14 were present in all three species with a relative abundance values identical under fed and unfed conditions, 2) C16 and C14 displayed major differences between the three studied species, 3) C16 to C14 ratios yielded a range of values, which were significantly different from each other ($p < 0.05$) (Table 1).

Table 2: C16:C14 Ratio defined as Species Differentiation Index (SDI) calculated for three adult female Rhipicephalus species independent of nutritional status

	R. annulatus	*R. sanguineus*	*R. Bursa*
C16:C14 Ratio	0.3 ± 0.2	27 ± 4	5 ± 2

Several studies demonstrated that *R. bursa* and *R. sanguineus* are closely related species based on their rRNA sequences (8). The similarities in the fatty acid profiles of the latter two species confirmed the similarities found in rRNA

and reinforced the utility of fatty acid profiles as a useful taxonomic and diagnostic tool in ticks. However, due to the difference in abundance of fatty acids C12, C22 and C24, they could be used as differentiation markers to *R. sanguineus* under fed and unfed conditions.

Various factors such as sex, age, physiological state, host, climate and geographic origin might result in variations of fatty acid composition, a well-documented phenomenon observed in bacteria and fungi (9-13). Therefore in future terms, an extensive study addressing the possible effects of the above mentioned factors is required, in order to elucidate the potential sources of variation in fatty acid composition. Collectively, our results indicate that fatty acid profile could serve as a valuable technique for tick taxonomy and lab diagnostics

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