Molecular Characterization of Some Equine Vector-Borne Pathogens and Identification of Their Vectors in Egypt

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Abstract

Background Equine vector borne diseases (EVBD) have been considered emerging and reemerging diseases transmitted by arthropods and most of these diseases have zoonotic concern. This study was designed to screen EVBD in equines and their vectors using molecular analyses and identify vectors by MALDI-TOF and molecular techniques.

Methods A total of 335 blood samples were collected from apparently healthy equines (320 from horses and 15 from donkeys) from Cairo and Beni-Suef provinces in Egypt. A total of 166 arthropods (105 sucking flies and 61 ticks) were collected from the same animals. MALDI-TOF and molecular techniques were used to confirm the findings of morphological identification of vector. Quantitative PCR and Standard PCR coupled with sequencing were performed in equines and vectors DNA for screening multiple pathogens.

Results MALDI-TOF and molecular techniques confirmed that *Hippoposca equina* (louse fly), *Rhipicephalus annulatus* (*Rh. annulatus*) and *Rh. microplus* ixodid ticks were found. In vectors, we identified *Anaplasma marginale* (*A. marginale*; 1.6%), *A. platys*-like (1.6%) and a new *Ehrlichia* sp. (4.9%) in *Rh. microplus*, while *Ehrlichia rustica* (*E. rustica*) was found in *Rh. microplus* and *Rh. annulatus*. Likewise, *Borrelia theileri* was identified in *Rh. microplus* (3.3%). For *H. equina*, *Anaplasma* and *Borrelia* sp. DNA were detected by qPCR only. In equines, *A. marginale* (0.6%), *A. ovis* (0.6%) and *Theileria ovis* (*T. ovis*; 0.6%) were found in donkeys. In horses, *T. equi* (1.2%) and a new *Theileria* sp. *Africa* (2.7%) were identified.

Conclusions For the first time, we reported here the presence of *Rh. microplus* as a competent tick for *Rh. annulatus* in Egypt using MALDI-TOF and molecular identification. To the best of our knowledge, we provided the first detection of different pathogens as *A. marginale*, *A. platys*-like, *E. rustica*, new *Ehrlichia* sp., *B. theileri* in *Rh. microplus*, *A. marginale*, *A. ovis* and *T. ovis* in donkeys and a new *Theileria* sp. *Africa* in horses in Egypt.

Background

Equine vector borne diseases (EVBD) have been considered emerging and reemerging diseases and most of these diseases have zoonotic concern [1]. Among haematophagic arthropods affecting *Equidae*, two major groups are to be mentioned as prominent infectious diseases vectors: Ixodid ticks (*Acari*) and hexapod *Diptera* (true flies) [2]. As a result of the vectors' parasitism, *Equidae* suffer from allergy, paralysis, myiasis and risk of transmission of various viral, bacterial, spirochetal and rickettsial diseases [1]. Since there is a distinct correspondence between the epidemiology of EVBD and the distribution of vectors [3], equines and their vectors play an important role in the maintenance and circulation of EVBD throughout the world [1, 4].

The *Anaplasmataceae* family includes intracellular bacteria such as *Anaplasma* and *Ehrlichia*, with a significant medical and veterinary importance [5, 6]. *Anaplasma* spp. are distributed worldwide especially

in tropical and subtropical Africa, and are responsible for granulocytic anaplasmoses and ehrlichioses in equine and canine populations and ruminants [7, 8]. These diseases are tick-borne and occur during spring and autumn seasons regarding tick activity [9]. Equine anaplasmosis (EA) has been reported in most of European countries, but the prevalence and incidence of EA in African countries, including Egypt, is limited [10].

The genus *Borrelia* includes pathogenic spirochaetes, which cause relapsing fevers and Lyme borreliosis [11]. Lyme disease is transmitted by hard ticks, while the relapsing fever borreliosises are transmitted by soft ticks [12, 13]. Equine borrelioses (EB) are characterized by shifting leg lameness, generalized stiffness, muscular weakness, lethargy and behavioral abnormalities [14]. Due to the neuromuscular and musculoskeletal effects of EB, it has been included in differential diagnosis in cases of lameness and poor performance in sport horses [15]. In Egypt, data regarding the incidence and prevalence of EB and their vectors are absent. *Borrelia burgdorferi* was detected in ticks [16, 17] and *Borrelia theileri* was detected in *Rhipicephalus annulatus* [17]. The *Borrelia flaB* and 16S rRNA genes were used for *Borrelia* detection [18].

Equine piroplasmosis (EP) is a tick-borne disease which is endemic in Europe, Asia, Russia, Africa and USA [19]. EP is caused by one of the hemoprotozoan parasites; *Theileria equi* and *Babesia caballi* [3, 20]. EP is characterized by fever, hemoglobinuria, jaundice, ventral edema, pale mucous membranes, anemia, weakness, lethargy, mild colic, abortion in mares, and death can occur in the acute phase of the infection [21, 22]. The mortality rate for *B. caballi* is 10%, while it reaches 50% for *T. equi* [3, 23]. The recovery from infection is possible, but recovered horses may become asymptomatic carriers in case of *T. equi*, while *B. caballi* is generally self-limited up to 4 years [24]. For these reasons, the equid movement across borders may be restricted [25]. Subsequently, EP is considered one of the biggest problems in international equid trade [19, 26].

In Egypt, the most prevalent Ixodid tick genera infesting equines are *Hyalomma* and *Rhipicephalus*. The genus *Hyalomma* includes *H. excavatum*, *H. dromedarii* and *H. marginatum* that are commonest species that infest equines [27, 28]. Besides, the genus *Rhipicephalus* (formerly *Boophilus*) includes *Rh. Annulatus*, which mainly infests cattle and is rarely found on equines [29]. The common dipteran infesting equines is the louse fly *Hippobosca equina* [1]. Recently, changes in tick distribution and introduction of exotic ticks with infectious agents into previously unaffected areas have been recorded [30–32]. Therefore, vector identification is essentially important for the epidemiological mapping of vectors and vector-borne diseases [33, 34]. Recently, the proteomic approach was applied for vector identification, which is called matrix-assisted laser desorption/ ionization time-of-flight mass spectrometry (MALDI-TOF MS) [35, 36]. MALDI-TOF successfully identified vectors through the spectra obtained from fresh, frozen or even alcohol preserved vector leg specimens, allowing to avoid difficulties of morphological taxonomy for identification due to damaged samples or immature stages [36–39]. Furthermore, this approach has been characterized by time saving, low cost and applicability on large scale studies when compared to molecular identification [40, 41].

The availability and significant increase in DNA sequence data due to the high sensitivity and accuracy of molecular techniques has attracted the interest of evolutionary researchers to more accurately identify and characterize previously detected and/or novel species and/or genotypes. Moreover, studying phylogenetic inferences and epidemiology of vector-borne pathogens contributes to the knowledge needed for disease control and prevention. In Egypt, reports for epidemiology of EVBD have been limited, except few reports regarding EP [20]. Therefore, the aim of this study was to screen Egyptian Equidae and their vectors for EVBD such as *Piroplasma*, *Anaplasma*, *Borrelia*, *Rickettsia*, *Coxiella burnetti* and *Bartonella* using molecular analyses. In addition, equine vectors were identified using MALDI-TOF and molecular techniques.

Materials & Methods

Animals and blood sampling

This study is a cross-sectional study and included a total of 335 apparently healthy *Equidae* (320 horses and 15 donkeys) using a convenience sampling strategy. Animals were examined for the presence of vectors on different parts of their body. Blood samples were collected from jugular vein in a sterile EDTA tube from different localities of Capital Cairo and Beni-Suef province in Egypt, between 2016 and 2017. All blood samples were stored at -20 °C until DNA extraction for molecular investigations. In addition, data for each animal were recorded by sex, breed, age, health status and vector infestation.

Vectors collection

A total 166 arthropods (105 sucking flies and 61 ticks) were collected from equines from different localities, as mentioned above. All vectors (flies and ticks) have been carefully collected to avoid physical damage. The vectors collected from the same animal were counted and pooled in one tube containing 70% ethanol and transfer to the lab for morphological, molecular and MALDI-TOF MS analyses.

Vectors Identification

1- Morphological Identification

Vector identification and gender detection were performed under microscope of X56 (Zeiss Axio Zoom.V16, Zeiss, Marly le Roi, France) using taxonomic identification keys [1, 42–44].

2- MALDI-TOF MS Identification

2.1. Sample preparation:

Ticks and sucking flies which reserved in 70% ethanol were rinsed twice in distilled water for 1 min then dried by sterile filter paper [36]. The legs of arthropod were cut by sterile scalpel, put in a sterile 1.5 ml Eppendorf and were then incubated at 37 °C overnight to evaporate any alcohol residue [38, 39]. The rest

of sample was cut longitudinally in two parts; one half was dissected into small species for molecular purpose and the remaining half was stored at − 20 °C as backup for any additional investigation.

2.2. Sample Homogenization and loading on MALDI-TOF target plates:

On the cut-off legs, a nip of glass powder (Sigma, Lyon, France) was added in addition 30 µl of a mix 70% (v/v) formic acid and 50% (v/v) acetonitrile (Fluka, Buchs, Switzerland) [36]. The legs were crushed and homogenized by a TissueLyserII device (Qiagen, Hilden, Germany) with 30 movements per second for 1 min and repeated three times [45]. After centrifugation of homogenized arthropod legs (at 2000 g for 30 sec), 1 µl of the supernatant of extracted protein was dropped onto a spot of MALDI-TOF polished steel plate (Bruker Daltonic, Wissembourg, France) in quadruplicate [45]. At room temperature, the plate was left to dry and then each spot covered with 1 µl of CHCA matrix solution that composed of saturated α-cyano-4-hydroxycynnamic acid (Sigma, Lyon. France), 50% acetonitrile (v/v), 2.5% trifluoroacetic acid (v/v) (Aldrich, Dorset, UK) and HPLC-grade water [41]. After drying, the plate was loaded into the Microflex LT MALDI-TOF MS apparatus (Bruker Daltonics, Germany) for analysis. Matrix solution was loaded in duplicate onto each MALDI-TOF plate with or without a bacterial test standard (Bruker protein Calibration Standard I) to control loading on the MS target plate, matrix quality and MALDI-TOF apparatus performance [45].

2.3. MALDI-TOF MS parameters:

The protein mass profiles of each sample were obtained using a Microflex LT spectrometer with the Flex Control software (Bruker Daltonics) with special parameters recommended by [36]. The spectra obtained were visualized by the Flex Analysis 3.3 software and transferred to ClinPro Tools version v.2.2 and MALDI-Biotyper v.3.0 (Bruker Daltonic, Germany) for analysis [37].

2.4. Spectra analysis and Reference database creation:

For spectra analysis, ClinPro Tools v2.2 and FlexAnalysis v3.3 software programs were used to evaluate the reproducibility of the MS spectra obtained from the same arthropods species and to assess intra-species specificity [47]. After morphological and molecular confirmation of arthropod identification, two to five high quality reproducible profiles with a high peak intensity of each species conserved in 70% ethanol were selected to serve as reference spectra [36, 48]. As for upgrade arthropod MALDI-TOF database, reference spectra were established by spectra at least 2 specimens per species of both genders using the algorithm MALDI-Biotyper software v3.0 (Bruker Daltonics) [39, 48].

2.5. Blind test:

All specimens were subjected to a blind test, except those used as reference spectra. The log score values (LSVs) were calculated by the MALDI-Biotyper software v.3.0 (Bruker Daltonics) to estimate the reliability of species identification. The LSVs ranged from 0 to 3; these correspond to the degree of similarity between the spectra submitted by blind test and the MS reference spectra in the database. The

identification was considered reliable with a LSV of at least 1.8 [36, 38]. The identification of blind tested samples was assessed by taking the highest LSVs associated with a spectrum quality.

Molecular techniques

1- DNA extraction:

Each dissected half of arthropod was put in a sterile 1.5 ml tube containing 200 µl of G2 lysis buffer and 25 µl of proteinase K (Qiagen, Hilden, Germany), cut in pieces and incubated at 56 °C overnight. 200 µl of supernatant was transferred into a new sterile tube after centrifugation of the mixture. DNA was extracted from 200 µl of each blood samples and arthropods using EZ1 DNA Tissue Kit (Qiagen) according to the manufacturer's instructions. The extracted DNA was stored at – 20 °C until use in molecular methods.

2- Molecular Identification of vectors:

Standard PCR was applied to confirm MALDI-TOF identification. Mitochondrial genes (CO1 and 16S rRNA gene) sequencing was used for the identification of flies and tick species through DNA automated thermal cycler (Applied Biosystem, Paris, France) under the same condition as previously described (Table 1) [48, 49]. PCR products were purified and sequenced as mentioned earlier [48]. The obtained sequences were assembled and corrected by ChromasPro software (ChromasPro 1.7, Technelysium Pty Ltd., Tewantin, Australia), and blasted against the reference sequences available in GenBank (http://blast.ncbi.nlm.nih.gov). The obtained sequences of Egyptian vectors (ticks and flies) were submitted in GenBank.

Table 1

Primers and probes used for qPCR, Standard PCR and sequencing in this study.

Microorganisms	Targeted Sequence	Primers f, r (5'-3') and Probes p (6FAM–TAMRA)	References
Quantitative PCR primers and probes			
Piroplasmida	5.8S rRNA	f_AYYKTYAGCGRTGGATGTC r_TCGCAGRAGTCTKCAAGTC p_TTYGCTGCGTCCTTCATCGTTGT	[50]
Anaplasmataceae	23S rRNA (TtAna)	f_TGACAGCGTACCTTTTGCAT r_TGGAGGACCGAACCTGTTAC p_GGATTAGACCCGAAACCAAG	[51]
Rickettsia sp.	gltA (RKNDO3)	f_GTGAATGAAAGATTACACTATTTAT r_GTATCTTAGCAATCATTCTAATAGC p_CTATTATGCTTGCGGCTGTCGGTTC	[52]
Borrelia sp.	Internal transcribed spacer 16S RNA (Bor ITS4)	f_GGCTTCGGGTCTACCACATCTA r_CCGGGAGGGGAGTGAAATAG p_TGCAAAAGGCACGCCATCACC	[53]
Coxiella burnetii	Insertion Sequence (IS1111)	f_CAAGAAACGTATCGCTGTGGC r_CACAGAGCCACCGTATGAATC p_CCGAGTTCGAAACAATGAGGGCTG	[54]
Bartonella sp.	Internal transcribed spacer16S (BartoITS3)	f_GATGCCGGGGAAGGTTTTC r_GCCTGGGAGGACTTGAACCT p_GCGCGCGCTTGATAAGCGTG	[55]
Standard PCR and Sequencing primers			
Piroplasmida	18S rRNA	F1- GCGAATGGCTCATTATAACA F4-CACATCTAAGGAAGGCAGCA F3-GTAGGGTATTGGCCTACCG R3-AGGACTACGACGGTATCTGA	[50]
Anaplasmataceae	23S rRNA	f-ATAAGCTGCGGGGAATTGTC r-TGCAAAAGGTACGCTGTCAC	[51]

3- Molecular detection of pathogen DNA in equines and their vectors:

Screening of pathogen DNA by qPCR:

Quantitative PCR (qPCR) was performed in all extracted DNA samples (equines and their vectors) for multi-pathogen DNA screening using genus-specific primers and probes targeting the 5.8S rRNA gene of *Piroplasmida* [50], the 23S rRNA gene of *Anaplasmataceae* [51], the *gltA* gene *Rickettsia* sp. [52], the 16S rRNA gene *Borrelia* sp. [53], the IS1111 intergenic spacer for *C. burnettii* [54] and 16S-23S intergenic spacer for *Bartonella* sp. [55] (Table 1). The qPCR was applied using the CFX96 Real Time System (Bio-

Rad, Marnes-La-Coquette, France). The mixture of qPCR contained 10 µl of Eurogentec Probe PCR Master Mix (Erogentec, Liège, Belgium), 0.5 µM of primers and FAM-labeled probe, 5 µl of DNA template 3.5 µl sterile distilled water to complete the reaction volume to 20 µl. The negative controls (without any DNA) and positive controls (corresponding pathogen DNA) were added to each reaction to evaluate the reaction. The samples were measured positive with the cycle threshold (Ct) lower than 35 Ct [56].

Standard PCR and Sequencing

The positive qPCR samples were subjected to standard PCR and sequencing. For identification of Piroplasmida (*Thierleria* spp. and *Babesia* spp.), *Anaplasmataceae* (*Anaplasma* spp. and *Ehrlichia* spp.) and *Borrelia* spp. 1100 bp of the 18S rRNA gene, a 520 bp fragment of the 23S rRNA gene and a 1200 bp of 16S rRNA gene were used; respectively [50, 51, 53]. The PCR reactions were performed on a Thermocycler (Applied Biosystem, Paris, France) using the AmpliTaq Gold® 360 Master Mix (ThermoFisher Scientific, USA) according to the manufacturer's recommendations. Negative and positive controls were included in each reaction. The PCR products were visualized by electrophoresis on 1.5% agarose gel stained with SYBR® Safe (Invitrogen, USA) and examined and analyzed by Lab Image software (BioRad, Marnes-La-Coquuette, France).

The purification of PCR products was applied using NucleoFast 96 PCR plates (Macherey–Nagel, Düren, Germany), in accordance with the manufacturer's recommendations. The purified PCR products were sequenced using the BigDye Terminator Cycle Sequencing Kit (3130 × 1 Genetic Analyzer, ABI-PRISM). The sequences obtained were assembled and edited by ChromasPro software (ChromasPro 1.7, Technelysium Pty Ltd., Tewantin, Australia) and the corrected sequences were compared with the reference sequences available in GenBank by BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequences obtained of Egyptian EVB genotypes were recorded in GenBank.

Phylogenetic analyses

Multiple sequence alignments were performed between the obtained sequences and other reference sequences in GenBank using CLASTAL W in MEGA software version X [57]. The phylogenetic trees were inferred using the Maximum-Likelihood method and Tamura-Nei model with 500 bootstrap replicates in MEGA X software [57, 58].

Results

Morphological identification of vectors:

A total of 105 louse flies were collected from 311 horses from Cairo and morphologically identified as *H. equina* (70 males and 35 females; Fig. 1). We examined 15 donkeys and 9 horses from Upper Egypt province (Beni-Suef) and found 7 *Rh. annulatus* on 3 horses and 4 donkeys. Moreover, we found 54 *Rhipicephalus* sp. on 15 donkeys and 8 horses, whose morphological identification could be confirmed using molecular biology and MALDI-TOF techniques. The details of collected vectors are listed in Table 2.

Table 2

Information data from the animals studied and their arthropod vectors

Provinces Animals (Total)	Gender Male/Female	Breed Native/Foreign/Arabic	Ticks		Sucking Flies (Hippobosca equina) (M/F)
			Rh. annulatus (M/F)	Rh. microplus (M/F)	
Cairo Horses (311)	202/109	271/34/6	–	–	105 (70/35)
Beni-Suef Horses (9) Donkeys (15)	3/6 0/15	9/0/0 15/0/0	0/3 2/2	0/25 2/27	– –
Total	205/130 (335)	295/34/6 (335)	2/5 (7)	2/52 (54)	105

Molecular identification of vectors:

Molecular identification was applied on specimens selected for creation of reference MS spectra. Based on CO1 (for flies) and 16S rRNA genes (for ticks), standard PCR and sequencing were applied and succeeded in identifying sucking flies as *H. equina* and *Rhipicephalus* sp. as *Rh. annulatus* and *Rh. microplus*. The obtained sequences were compared against GenBank database with 99% identity to reference sequences. The obtained sequences of CO1 of *H. equina*, and 16S rDNA of *Rh. annulatus* and *Rh. microplus* were deposited in GenBank data base under the following accession numbers: MK737646, MK737648 and MK737647; respectively.

MALDI-TOF MS analyses and Validation of vectors identification by blind tests:

In-lab MS reference spectra database was essential for accurate identification of vectors specimens [39, 48]. After morphological and molecular confirmation, *H. equina* (Fig. 2), *Rh. annulatus* and *Rh. microplus* (Fig. 3) from Egypt were introduced as reference MS spectra into in-lab database. Once the database updated, a blind test was performed on the rest of arthropods. The Microflex LT MALDI-TOF MS enabled the identification of 99/105 (94%) for *H. equina* ($1.22 \leq LSVs \leq 2.48$), 7/7 (100%) for *Rh. annulatus* ($1.62 \leq LSVs \leq 2.72$) and 52/54 (96%) for *Rh. microplus* ($1.69 \leq LSVs \leq 2.81$).

Pathogens detection in vectors

All arthropods DNA samples were screened for the detection of pathogen DNA of *Piroplasma* sp., *Anaplasma* sp., *Rickettsia* sp., *Borrelia* sp., *C. burnetii* and *Bartonella* sp. The result revealed that DNA of *Anaplasma* and *Borrelia* sp. were detected in *Rhipicephalus* sp. collected from donkeys and *H. equina*

from horses. While, *Rhipicephalus* sp. collected from horses were free from any pathogen DNA (Table 3). All vectors were found to be free from *Piroplasma, Rickettsia, C. burnetii* and *Bartonella* infections.

Table 3
The prevalence of pathogens in Equines and their vectors.

Pathogens	Horses	Donkeys	Total prevalence in Equines	Rh. annulatus	Rh. microplus	Total prevalence in Ticks
Anaplasmataceae	–	4/15 (26.6%)	4/335 (1.2%)	1/7 (14.3%)	8/54 (14.8%)	9/61(14.7%)
Anaplasma marginale	–	2/15 (13.3%)	2/335 (0.6%)	–	1/54 (1.8%)	1/61 (1.6%)
Anaplasma ovis	–	2/15 (13.3%)	2/335 (0.6%)	–	–	–
Anaplasma platys	–	–	–	–	–	1/61 (1.6%)
Ehrlichia rustica	–	–	–	–	1/54 (1.8%)	4/61 (6.6%)
Ehrlichia sp.		–	–	1/7 (14.3%)	1/54 (1.8%)	3/61 (4.9%)
		–	–	–	3/54 (5.6%)	
		–	–		3/54 (5.6%)	
Borrelia sp.	–	–	–	–	2/54 (3.7%)	2/61(3.3%)
Borrelia theileri	–	–	–	–	2/54 (3.7%)	2/61(3.3%)
Piroplasmida	13/320 (4.1%)	2/15 (13.3%)	15/335 (4.5%)	–	–	–
Theileria equi	4/320 (1.3%)	–	4/335 (1.2%)	–	–	–
Theileria sp. Africa	9/320 (2.8%)	–	9/335 (2.7%)	–	–	–
Theileria ovis	–	2/15 (13.3%)	2/335 (0.6%)			

Anaplasma DNA were detected using qPCR based on 23S rRNA gene in 21/166 (12.7%), 16/54 (29.6%) in *Rh. microplus*, 1/7 (14.3%) in *Rh. annulatus* and 4/105 (3.8%) in *H. equina* vectors. We were able to amplify and sequence the rRNA portion of the *Anaplasmataceae* 23S rRNA gene in 9 of the 17 samples, all from *Rhipicephalus spp*. collected from donkeys. The Blast research reported the presence of two different genotype groups that were attributed to two genera of *Anaplasmataceae* (*Anaplasma* and *Ehrlichia*) in *Rhipicephalus* spp. A new *A. platys* genotype (GenBank: MN614105) was detected in one *Rh. microplus* with 100% (415/415) identity with *A. platys* genotype detected in cattle and sheep from the same locality of Beni Suef province (GenBank: MN626397 & MN626398) and clustered in the same clade (bootstrap value 99; Fig. 4). In addition, this genotype has 99% (409/415) identity with those of I detected in the blood of New Caledonian dogs (GenBank: KM021425). Another *Anaplasma* sp. that was detected in *Rh. microplus* was *A. marginale* genotype (GenBank: MN614106) with 100% (380/380) similarity to those of *A. marginale* detected in the blood of cattle from Algeria (GenBank: MH321194). Moreover, two *Ehrlichia* sp. with three different genotypes were found in *Rhipicephalus* sp., new genotype close to "*E. rustica"* was derived from one *Rh. microplus* (GenBank: MN614107) with 99% (413/415) identity to those of *E. rustica* found in *Amblyomma vargiegatum* from Côte d'Ivoire (GenBank: KT364330) and "*E. rustica"*

genotype was derived from two *Rh. microplus* and one *Rh. annulatus* (GenBank: MN614108 & MN614109; respectively) with 100% (415/415) identity to the same reference. Finally, a new potential *Ehrlichia* sp. was derived from three *Rh. microplus* (GenBank: MN614110) with 98% (404/413) similarity to those of Candidatus *E. urmitei* detected in *Rh. bursa* in France. In the phylogenetic tree, the sequence of this potential new *Ehrlichia* sp. clustered in separated and well supported branch (bootstrap value 55) with *E. ruminantium* (Fig. 4). The phylogenetic position of these genotypes was illustrated in Fig. 4.

For *Borrelia* sp., the result of qPCR revealed that 5 out of 166 were positive for *Borrelia* sp. (2 (3.7%) in *Rh. microplus* and 3 (2.9%) *H. equina*). Using 16S rRNA gene sequencing, we identified it as *B. theileri* that derived from two *Rh. microplus*. The result revealed that a new genotype of *B. theileri* (GenBank: MN619805) was identical to *B. theileri* previously detected in sheep and cattle from the same locality of Beni-Suef province (GenBank: MN621893 & MN621894). The phylogenetic position of this *B. theileri* genotype is illustrated in Fig. 5.

Pathogens detection in Equidae:

All blood samples were screened by qPCR to screen the presence of pathogen DNA of Piroplasmids, *Anaplasmataceae*, *Rickettsia* spp., *Borrelia* spp., *Coxiella burnetii* and *Bartonella* spp. DNA of *Piroplasmida* and *Anaplasma* spp. were detected in horses and donkeys, while blood samples were free from the other DNA pathogens (Table-3).

For *Anaplasma* sp., 33 out of 335 (9.8%; 28 horses and 5 donkeys) were positive qPCR system. We obtained good quality 23S rDNA sequences from only 4 samples, probably due to the low amount of DNA in the blood samples. Based on BLAST analyses, we identified two species of *Anaplasma*, a genotype of *A. ovis* was derived from two donkeys (GenBank: MN614104) with 100% (454/454) identity to *A. ovis* detected in sheep blood from Niger (GenBank: KY644694) and one detected in sheep and cattle blood of the same locality (GenBank: MN626392 & MN625933). Another species identified was *A. marginale*, identified in two donkeys (GenBank: MN614103) with 100% (455/455) identity to those detected in *Rhipicephalus bursa* collected from cattle in France (GenBank KY498335) and those detected in blood of cattle collected from Egypt (GenBank: MN625935). The phylogenetic position of these genotypes was shown in Fig. 4.

Piroplasmida DNA was detected in 27/335 animals (8.1%, 25 horses and 2 donkeys) on the basis of the 5.8S rRNA gene. As a result, PCR and sequencing of the 18S rRNA gene portion was performed and allowed the identification of 15 of the 27 qPCR positive samples. (4.5%, 13 in horses and 2 in donkeys). According to BLAST search, we identified three *Piroplasma* species: *T. equi*, a new potential *Theileria* sp. "*Africa"* and *T. ovis*. In horses, we identified two *Piroplasm* sp. (*T. equi* and *Theileria* sp. "*Africa"*). By Blast research, we obtained two genotypes of *T. equi*, one genotype was derived from one horse and another genotype derived from three horses (GenBank: MN625897 & MN625898) with 100% (922/922) identity to those of *T. equi* detected in horses from Israel (GenBank: MK063843 and MK063842; respectively). Moreover, we found four genotypes of *Theileria* sp. "*Africa"* (GenBank: MN625900, MN625901, MN625902 & MN625903), that clustered in a separate clade with a good bootstrap support with the other

Theileria sp. "*Africa"* previously detected in African horses (Fig. 6). By BLAST analyses, one genotype from horse was considered to be a new genotype with 99% (917/918) identity to *T. equi* detected in horse blood from Brazil (GenBank:MG052913). Three other genotypes were identified as *T. equi* with 100% (923/923) identity to those detected in horse blood from Turkey and Sudan (GenBank: MG569896, MG569893 and AB515309; respectively). In donkeys, *T. ovis* shared 100% (919/919) identity with sheep and buffalo previously detected in the same province in Egypt (GenBank: MN625886 & MN625887).

Discussion

Equidae are used in many beneficial activities for human such as police services, agriculture and pharmaceutical purposes, in addition to competitive and non-competitive leisure pursuits [59]. Generally, *Equidae*, especially donkeys, play a significant role in the transmission of vector borne diseases by acting as a domestic reservoir and carrying vectors to a broad host range or even to human [1]. Recently, the spectrum of EVBD has increased and drawn the attention of veterinarians and clinicians to diseases such as, piroplasmoses, anaplasmoses, borrelioses, rickettsioses, bartonelloses and Q fever [33]. In addition, advances in molecular biology tools and the availability of DNA sequence data facilitate the detection of new pathogen species and even genotypes [60]. The present study summarized epidemiological and entomological data on the prevalence of EVBDs infecting *Equidae* and their vector in two regions of Egypt (Capital Cairo and Beni-Suef province). Besides, equine arthropod parasites were identified by MALDI-TOF and molecular techniques.

Hyalomma and *Rhipicephalus* Ixodid ticks and the dipteran *H. equina* are the most common vectors infesting equines [1, 27, 28]. In this study, the morphological identification of vectors revealed the presence of *Rhipicephalus* sp. as ixodid ticks collected from horses and donkeys of Beni Suef province. Also *H. equina* was morphologically identified from horses in Cairo. In support of these morphological identification results, previous studies have reported the presence of *Rhipicephalus* sp. (especially *Rh. annulatus*) in Egypt as the main ixodid ticks infesting cattle and that may infest equines [29] and *H. equina* was louse fly of horses [61]. The morphological similarities at both intra- and inter-species level limit the worth of morphological taxonomic key such as in *Rhipicephalus* sp. [62–64]. Therefore, MALDI-TOF MS was applied to identify *Rhipicephalus* sp. and *H. equina*. MALDI-TOF MS confirmed that we have two different species of *Rhipicephalus*: *Rh. microplus* and *Rh. annulatus*. The molecular identification confirmed MALDI-TOF MS results. CO1 of *H. equina* and 16S rRNAs of *Rh. annulatus* and *Rh. microplus* were used for the identification and were deposed in GenBank (MK737646, MK737648 and MK737647; respectively). The percentages of coverage and identity between sequences obtained from the same species were 99%. After molecular confirmation, MS spectra of *H. equina*, *Rh. annulatus* and *Rh. microplus* were deposed in the database as reference MS spectra, and then, a blind test was applied on all vector samples. The majority of *Rhipicephalus* sp. were identified as *Rh. microplus* that were collected from horses and donkeys from Beni-Suef province. To the best of our knowledge, *Rh. microplus* has never been reported in Egypt or even in North Africa. *Rh. microplus* is a Southeast Asian tick, introduced into Southeast Africa by cattle from Madagascar [42]. In 2007, it was reported for the first time as an invasive tick in West Africa [65]. Then, it spread and was reported in other West African countries as Togo and Burkina Faso [66], Benin [67], Mali [39, 66] and Côte d'Ivoire [60, 68]. That indicates the rapid spread of *Rh.*

microplus through the African countries and the risk of its invasion to North Africa. The change in tick distribution and introduction of exotic ticks might be attributed to climatic change, host availability and animal movements [30, 31].

Family Anaplasmataceae includes two significant genera, *Anaplasma* and *Ehrlichia*, which can cause significant infections in a wide range of animal hosts and humans. These infections are mainly transmitted by ticks [69]. In the present study, the overall prevalence of Anaplasmataceae infection in *Rhipicephalus* sp. collected from *Equidae* was 14.7%. This study reports for the first time a novel potential *Anaplasma* sp. in *Rh. microplus* in Egypt. This *Anaplasma* sp. was genetically close to canine *A. platys* with 99% homology, so that it was commonly named *A. platys*-like. The phylogenetic tree revealed that our *A. platys*-like was grouped in the same clade with canine *A. platys* (bootstrap value 89; Fig. 4). However, it forms a separate clade with *A. platys*-like detected in cattle and sheep from Beni Suef province where *Rh. microplus* was collected from donkeys (**Abdullah et al., unpublished**). As far as we know, the presence of *A. platys*-like has never been reported in Africa in *Rh. microplus*. Recently, *A. platys*-like was reported in *Rh. microplus* in China [70] and Pakistan [71]. Later, *A. platys*-like was also identified in different animal hosts other than dogs as cattle in Italy [72], Algeria [73] and Tunisia [74], and sheep and goat in South Africa [75] and Senegal [76]. Similarly, our study is the first to report the presence of *A. marginale* in *Rh. microplus* in Egypt. *A. marginale* has previously been reported in *Rh. microplus* in Côte d'Ivoire [60], Ecuador [77], India [78] and Pakistan [79]. As for genus *Ehrlichia*, we recorded for the first time two different genotypes of "*E. rustica"* in both *Rh. microplus* and *Rh. annulatus* in Egypt. One genotype of "*E. rustica"* was identified in *Rh. microplus* and *Rh. annulatus* with 100% homology to those of *E. rustica* found in *Amblyomma vargiegatum* from Côte d'Ivoire, and another genotype was identified in *Rh. microplus* only with 99% homology to the same reference [60]. Moreover, we have also identified a new potential *Ehrlichia* sp. in three *Rh. microplus* with 98% similarity to those of Candidatus *E. urmitei* detected in *Rh. bursa* from France (Fig. 4). The sequence of this potential *Ehrlichia* sp. clustered in a separated clade with *E. ruminantium* (bootstrap value 55; Fig. 4). As a result, we had a new potential *Ehrlichia* sp. in *Rh. microplus* and *E. rustica* in *Rh. annulatus* that had never been reported before in Egypt. Interestingly, these potential new species were identified in three different regions in the world (France, Côte d'Ivoire and Egypt) and from different tick species (*Rhipicephalus*, *Amblyomma*, and *Hyalomma* sp.) [60]. Thus, *Rh. microplus* could be an alternative vector for *Anaplasmataceae* alongside *Rh. annulatus* in Egypt, and there is a risk of transmission of other potential new vector-borne diseases and this should be evaluated in future studies.

In Africa, most of the *Borrelia* species were detected in soft ticks, such as *Ornithodoros* sp. which is the main vector [80]. To date in Africa, *Borrelia* sp. was identified in hard ticks (*Amblyomma* and *Rhipicephalus* sp.) in Ethiopia [81, 82], Mali [18], Côte d'Ivoire [60], Egypt [17], Madagascar [83] and Ecuador [77]. In the present study, a new potential *B. theileri* was identified in two *Rh. microplus* with 3.3% infection rate. The obtained sequence was 99% identical to *B. theileri* found in *Rh. geigyi* in Mali [18]. Likewise, the phylogenetic analysis revealed that a new genotype of *B. theileri* was clustered in the same clade with *B. theileri* detected in sheep and cattle from the same locality of Beni-Suef province (GenBank: MN621893 & MN621894; **Abdullah et al., unpublished**). *B. theileri* in *Rh. microplus* has been reported in

Madagascar [83], Ecuador [77], Brazil [84] and Argentina [85]. Thus, this is the first time that *B. theileri* has been detected in *Rh. microplus* in Egypt. It had previously been identified in *Rh. annulatus* [17].

Regarding *H. equina*, *Anaplasma* and *Borrelia* sp., DNAs were detected by qPCR. However, we were unable to amplify and sequence these samples, which could be attributed to the high sensitivity of qPCR compared to standard PCR, or to the low concentration of pathogenic DNA in fly tissues. Kowal and his colleagues [86] reported the role of Hippoboscids in the transmission of bacterial pathogens such as *Anaplasma* and *Bartonella*. Moreover, Boucheikhchoukh and his colleagues [87] detected *Bartonella* and *Wolbachia* sp. in *H. equina*.

In *Equidae* blood, we reported Anaplasmataceae DNA in donkeys and Piroplasimda DNA in horses. For Anaplasmataceae, the overall prevalence of anaplasmoses in donkeys was 26.6%, while horses were found free from *Anaplasma* sp. This result was in accordance with [60], who did not find any *Anaplasma* in horses. *A. ovis* and *A. marginale* were the common *Anaplasma* pathogens of sheep and cattle; respectively [76]. However, in our study, we found *A. ovis* and *A. marginale* in donkeys. *A. ovis* shared 100% identity to those of *A. ovis* in sheep and cattle blood from the same locality of Beni-Suef province (GenBank: MN626392 & MN625933), as well as in the blood of sheep from Niger (GenBank: KY644694). Another *Anaplasma* sp. was *A. marginale* that shared 100% similarity with those of *A. marginale* detected in blood of cattle collected from the same locality of Beni-Suef province (GenBank: MN625935; **Abdullah et al., unpublished**). To the best of our knowledge, *A. ovis* and *A. marginale* have never been reported yet in donkeys in Egypt and even Africa. Also, *A. marginale* has been reported in donkeys in Pakistan [88]. Therefore, donkeys should be involved in the epidemiology of tick-borne pathogens and other associated agents such as Anaplasmoses of health importance.

EP is a protozoan disease caused by *T. equi* and *B. caballi* [3, 20]. Our study reported an overall prevalence of EP at 4.5% (1.2% for *T. equi*, 2.7% for *Theileria* sp. "*Africa"* and 0.6% for *T. ovis*), but we did not detect *B. caballi*. This might be attributed to self-limiting of *B. caballi* infection and the lifetime persistence of *T. equi* [89]. In this study, two genotypes of *T. equi* were pooled in a separate clade with *T. equi* that has already been reported in horses in America [90] and Israel [91]. Yet, a new potential *Theileria* sp. "*Africa"* genotypes were clustered in a separate clade of a good bootstrap support with the other *Theileria* sp. "*Africa"* previously detected in African horses in Senegal and Chad (Fig. 6) [50]. As far as we know, *Theileria* sp. "*Africa"* has never been reported yet in Egypt. *T. ovis* was detected in donkeys with a prevalence rate of 0.6%, representing its first detection in donkeys in Africa. Recently, *T. ovis* was reported in horses and donkeys in Turkey [92]. In the last decade, several studies have reported the existence of other piroplasmid species in horses and donkeys and have reduced the host specificity of piroplasmids [93, 94].

Conclusion

In conclusion, the present study summarized the epidemiological and entomological data of the prevalence of EVBD infecting equines and their vector in two regions of Egypt (Cairo and Beni-Suef

province). We reported, for the first time, the presence of *Rh. microplus* as a competent tick for *Rh. annulatus* in Egypt using MALDI-TOF and molecular identification, which increases the risk of transmission of other potential new vector-borne diseases, and this should be assessed in future studies. Also, we reported the first detection of *A. marginale*, *A. platys*-like, *"E. rustica"*, new *Ehrlichia* sp., *B. theileri* in *Rh. microplus*, *A. marginale*, *A. ovis* and *T. ovis* in donkeys and a new *Theileria* sp. "*Africa" in* horses in Egypt. Therefore, equines, especially donkeys, should be involved in the epidemiology of tick-borne diseases as they serve as reservoirs for these emerging and remerging pathogens to other animals.

Declarations

Ethical approval and Consent to participate

This study was approved by the Medical Research Ethics Committee at the National Research Centre, Egypt under the number 19059.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request. In addition, the obtained sequences in this study were submitted to the GenBank database under their accession numbers.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

All authors were involved in the design of the study. HHAMA, DA and TKF collected blood and vector samples. HHAMA, DA, KNA and SA identified fly and ticks morphologically. HHAMA and DA shared in DNA extraction. HHAMA, SA, DR and OM shared in molecular protocols and data analysis. HHAMH, PP

and OM shared in MALDI-TOF protocols and data analysis. HHAMH wrote the first draft of the manuscript. All authors revised and approved the last version of the manuscript.

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Unsectioned Tables

Figures

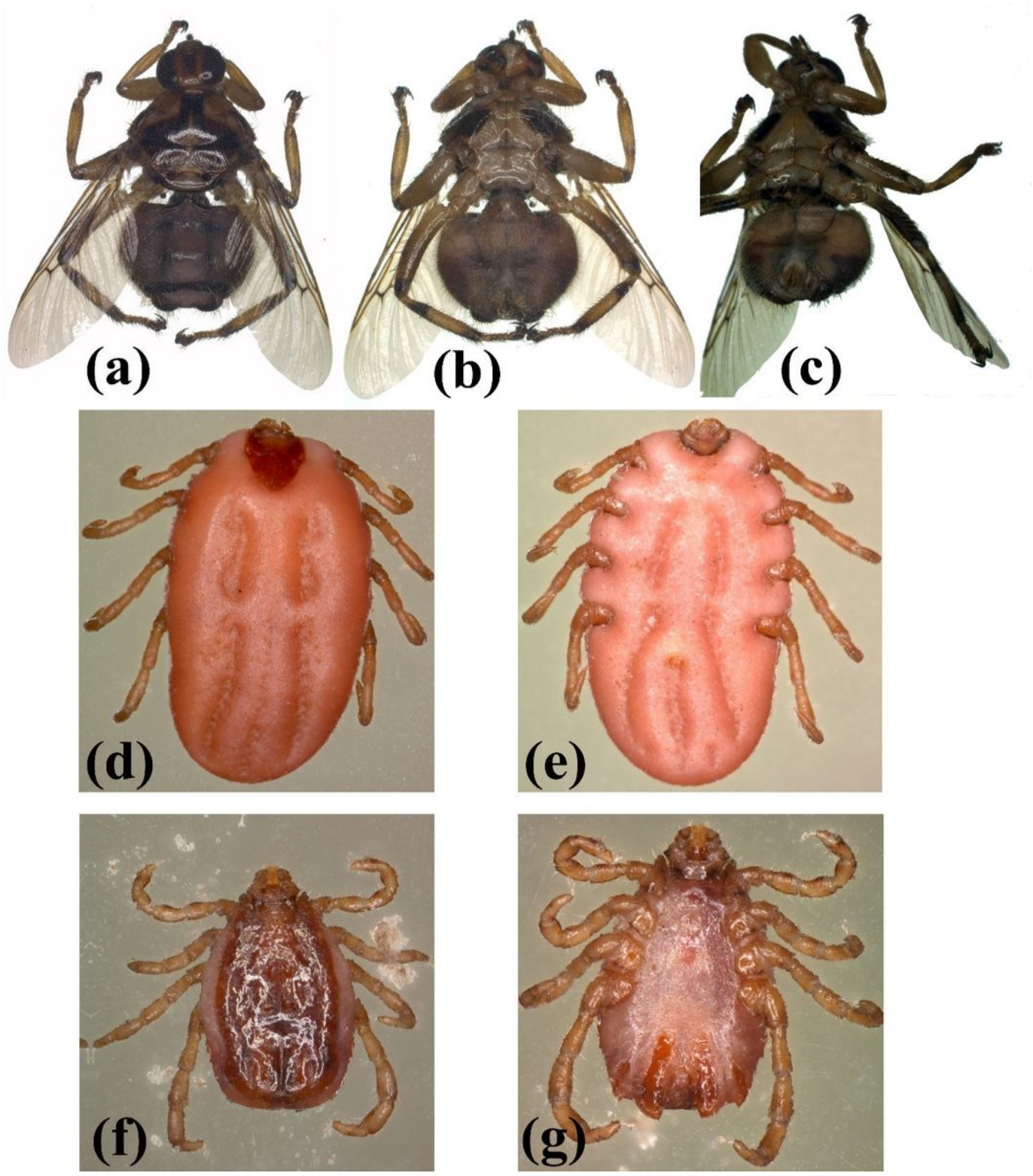

Figure 1

The arthropod vectors were collected from horses and donkeys, the louse fly Hippobosca equina (a-c) and ixodid tick Rhipicephalus (formerly Boophilus) annulatus (d-g). a) dorsal view of H. equina, b) ventral view of H. equina female, c) ventral view of H. equina male, d) dorsal view of R. annulatus female, e) ventral view of R. annulatus female, f) dorsal view of R. annulatus male and g) ventral view of R. annulatus male.

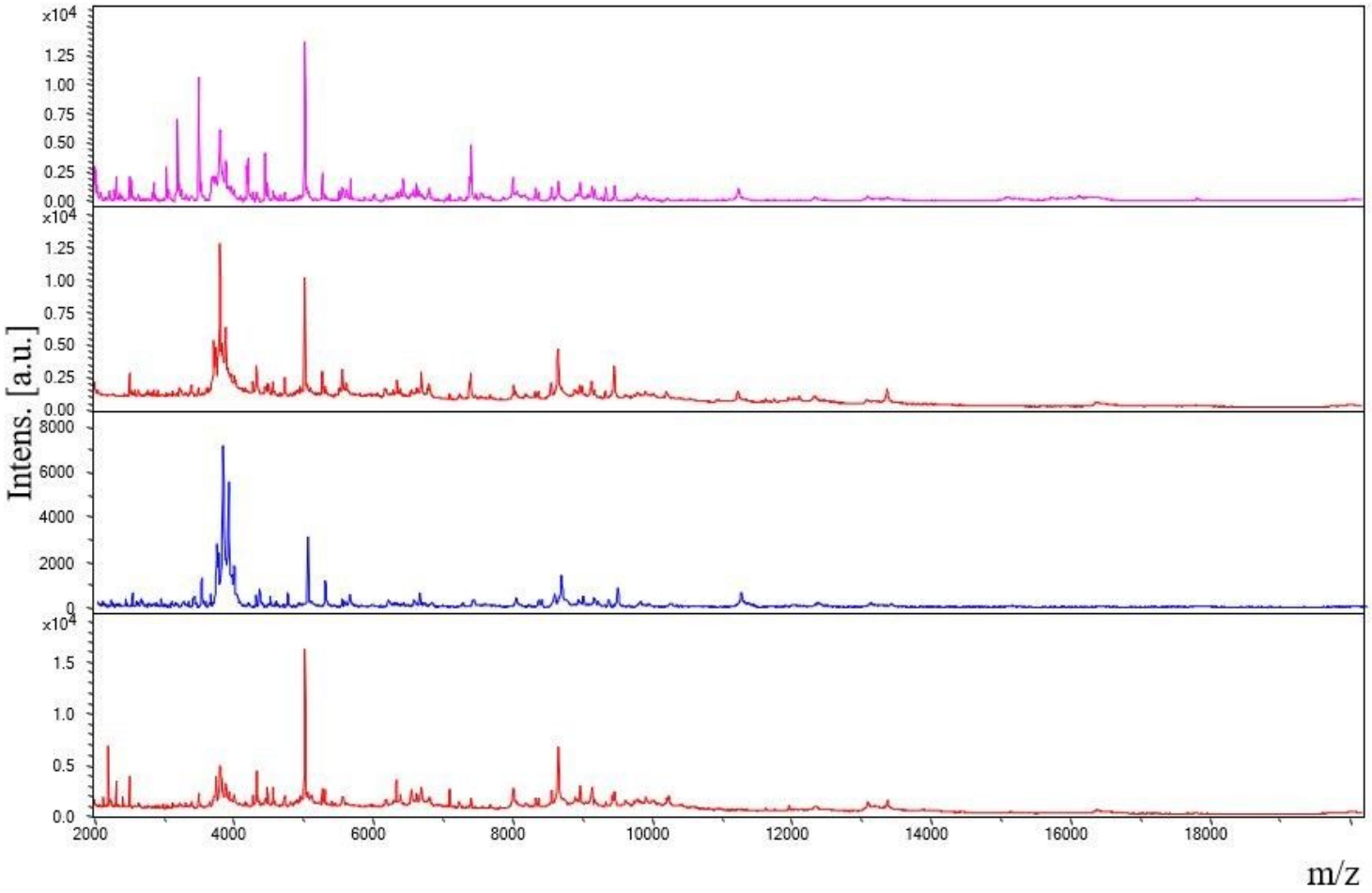

Figure 2

Mass Spectrometer profiles of Hippobosca equina aligned using Flex Analysis v3.3 software. [Intens] Intensity; [a.u] arbitrary units; [m/z] mass-to-charge ratio. A random color was used for the spectrum of each specimens.

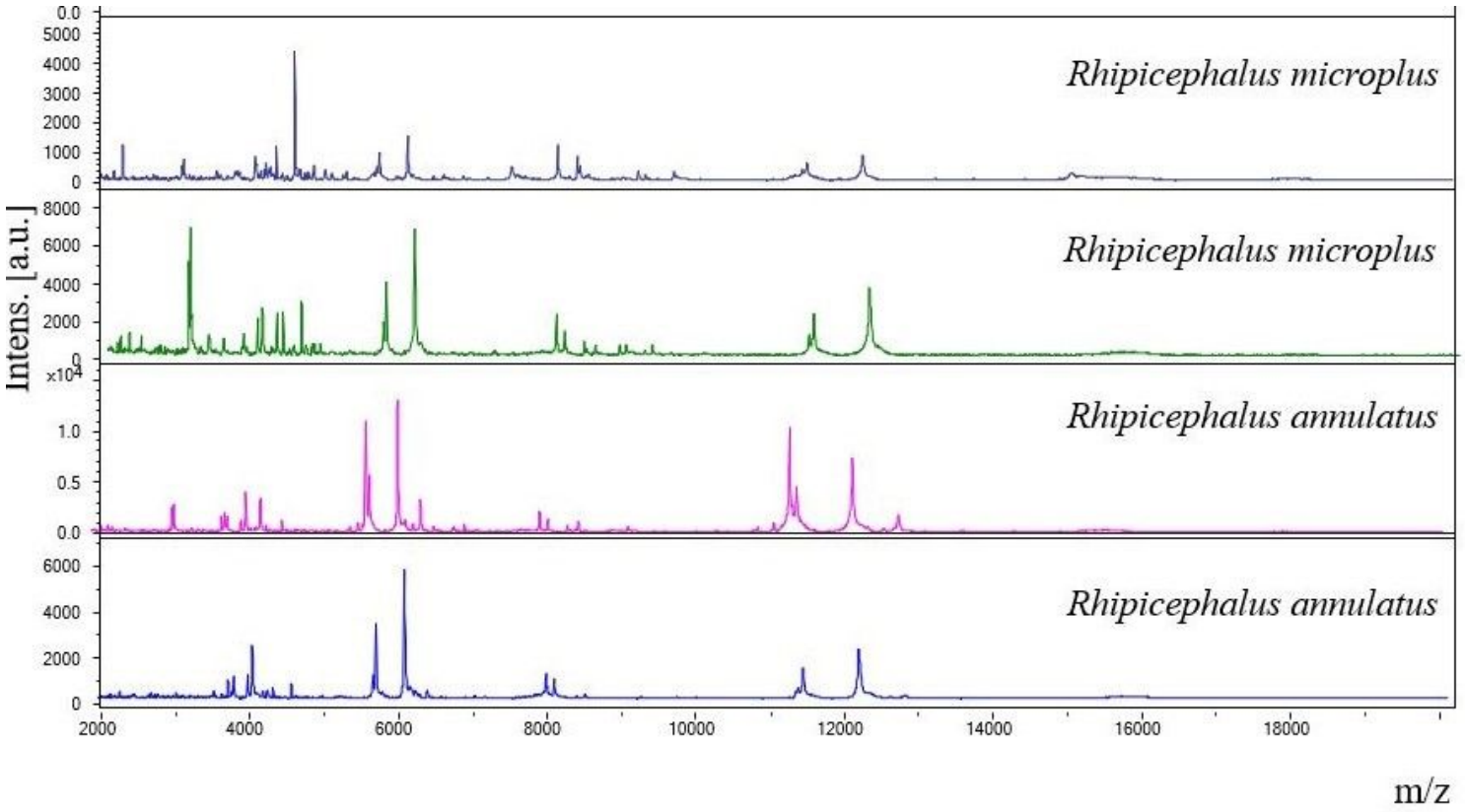

Figure 3

Mass Spectrometer profiles of Rhipicephalus sp. (Rh. annulatus and Rh. microplus) aligned using Flex Analysis v3.3 software. [Intens] Intensity; [a.u] arbitrary units; [m/z] mass-to-charge ratio. A random color was used for the spectrum of each specimens.