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1	Molecular characterization of some equine vector-borne diseases and
2	associated arthropods in Egypt
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13	vector-borne diseases.

15 Abstract

16 Equine vector-borne diseases (EVBDs) are emerging and re-emerging diseases, and most of them are zoonotic. This study aimed to investigate EVBDs in equines and associated 17 arthropods (ticks and flies) from Egypt using molecular analyses, in addition to a preliminary 18 characterization of associated ticks and flies by the matrix-assisted laser desorption/ionization 19 time of flight (MALDI-TOF) and molecular techniques. In this study, 335 blood samples were 20 21 obtained from equines that appeared to be in good health (320 horses and 15 donkeys) in Cairo and Beni Suef provinces, Egypt. From the same animals, 166 arthropods (105 sucking 22 flies and 61 ticks) were collected. Ticks and flies were preliminary characterized by the 23 MALDI-TOF and molecular tools. Quantitative PCR (qPCR) and standard PCR coupled with 24 sequencing were performed on the DNA of equines, ticks, and flies to screen multiple 25 pathogens. The MALDI-TOF and molecular characterization of arthropods revealed that louse 26 fly (Hippobosca equina) and cattle tick (Rhipicephalus annulatus) infesting equines. 27 Anaplasma platys-like (1.6%), Anaplasma marginale (1.6%), Candidatus Ehrlichia rustica 28 29 (6.6%), a new Ehrlichia sp. (4.9%), and Borrelia theileri (3.3%) were identified in R. annulatus. Anaplasma sp. and Borrelia sp. DNAs were only detected in H. equina by qPCR. 30 A. marginale, Anaplasma ovis, and Theileria ovis recorded the same low infection rate (0.6%) 31 32 in donkeys, while horses were found to be infected with Theileria equi and a new Theileria sp. Africa with recorded prevalence rates of 1.2% and 2.7%, respectively. In conclusion, 33 different pathogens were first detected such as A. platys-like, Candidatus E. rustica, and a 34 new Ehrlichia sp. in R. annulatus; A. marginale, A. ovis, and T. ovis in donkeys; and a new 35 Theileria sp. "Africa" in horses. 36

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40 **1. Introduction**

Equine vector-borne diseases (EVBDs) are emerging and re-emerging diseases, and 41 most of them are zoonotic (Onmaz et al., 2013). Among hematophagous arthropods affecting 42 Equidae, two major groups are to be mentioned as prominent infectious diseases vectors: 43 ixodid ticks (Acari) and hexapod Diptera (true flies) (Kaufman et al., 2008). As a result of the 44 vectors' parasitism, Equidae suffers from allergy, paralysis, myiasis, and the risk of 45 46 transmission of various viral, bacterial, spirochetal, and rickettsial diseases (Onmaz et al., 2013). Since there is a distinct correspondence between the epidemiology of EVBDs and the 47 distribution of vectors (Wise et al., 2013), equines and their vectors play a significant role in 48 the maintenance and circulation of EVBDs throughout the world (Štefanidesová et al., 2008 49 Onmaz et al., 2013). 50

51 The Anaplasmataceae family includes intracellular bacteria such as Anaplasma and Ehrlichia, with a significant medical and veterinary importance (Dumler et al., 2001; Pruneau 52 et al., 2014). Anaplasma spp. are distributed worldwide especially in tropical and subtropical 53 54 Africa and are responsible for granulocytic anaplasmosis in equine as well as in canine populations and ruminants (Laus et al., 2013; Vichova et al., 2014). These diseases are tick-55 borne and occur during spring and autumn seasons regarding tick activity (Bown et al 2003). 56 57 Equine anaplasmosis (EA) has been reported in most of European countries, but the prevalence and incidence of EA in African countries, including Egypt, are limited (Saleem et 58 al., 2018). 59

The genus *Borrelia* includes pathogenic spirochetes, which cause relapsing fevers and Lyme borreliosis (Haitham et al., 2013). Lyme disease is transmitted by hard ticks, while relapsing fever borreliosis is transmitted by soft ticks (Vial et al 2006; Trape et al., 2013). Equine borreliosis (EB) is characterized by shifting leg lameness, generalized stiffness, muscular weakness, lethargy, and behavioral abnormalities (Divers et al., 2018). Due to the neuromuscular and musculoskeletal effects of EB, it has been included in differential

diagnosis in situations of lameness and poor performance in sport horses (Swinebroad, 2018).
Data regarding the incidence and prevalence of EB and their vectors are absent in Egypt. *Borrelia burgdorferi* was detected in ticks (Adham et al., 2010; Hassan et al., 2017), and *Borrelia theileri* was detected in *Rhipicephalus annulatus* (Hassan et al., 2017). The *Borrelia flaB* and 16S rRNA genes were used for *Borrelia* detection (McCoy et al., 2014).

Equine piroplasmosis (EP) is a tick-borne disease that is endemic in Europe, Asia, 71 Russia, Africa, and the United States (Kouam et al., 2010). EP is caused by one of the 72 hemoprotozoan parasites: Theileria equi and Babesia caballi (Wise et al., 2013, Mahmoud et 73 al., 2016). EP is characterized by fever, hemoglobinuria, jaundice, ventral edema, pale 74 75 mucous membranes, anemia, weakness, lethargy, mild colic, abortion in mares, and death (Rothschild and Knowles, 2007; Zobba et al., 2008). The mortality rate for *B. caballi* is 10%, 76 while it reaches 50% for T. equi (Tamzali, 2013; Wise et al., 2013). The recovered horses may 77 become asymptomatic carriers in case of T. equi, whereas B. caballi is usually self-limiting 78 for up to 4 years (Rothschild, 2013). For these reasons, the equid movement across borders 79 may be restricted (Knowles, 1996). Subsequently, EP is considered one of the biggest 80 problems in the international equid trade (Kouam et al., 2010; Scoles and Ueti, 2015). 81

In Egypt, the most prevalent ixodid tick genera infesting equines are Hyalomma and 82 83 Rhipicephalus. The genus Hyalomma includes Hyalomma excavatum, Hyalomma dromedarii, and *Hyalomma marginatum* that are the commonest species that infest equines (Abdel-Shafy, 84 1994; Abdel-Shafy, 2000). Moreover, the genus Rhipicephalus (formerly Boophilus) includes 85 *R. annulatus*, which mainly infests cattle and is rarely found on equines (Abdel-Shafy et al., 86 2013). The common dipteran infesting equines is the louse fly Hippobosca equina (Onmaz et 87 al., 2013). Recently, changes in tick distribution and introduction of exotic ticks with 88 infectious agents into previously unaffected areas have been recorded (Estrada-Pena and 89 Venzal, 2007, Estrada-Pena, 2008; Chitimia-Dobler et al., 2016). Therefore, arthropod 90 identification is essentially significant for the epidemiological mapping of arthropods and 91

arthropod-borne diseases (Dantas-Torres et al., 2013; Lu et al., 2013). Recently, the proteomic 92 93 approach was applied for arthropod identification, which is called matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) (Dvorak et al., 94 2014; Kumsa et al., 2016). The MALDI-TOF successfully identified arthropods through the 95 spectra obtained from fresh, frozen, or even alcohol-preserved vector leg specimens, avoiding 96 difficulties of morphological taxonomy for identification due to damaged samples or 97 immature stages (Karger et al., 2012; Yssouf et al., 2013; Kumsa et al., 2016; Diarra et al., 98 2017). Furthermore, this approach has been characterized by time saving, low cost, and 99 applicability on large-scale studies compared with molecular identification (Araya-Anchetta 100 101 et al., 2015; Yssouf et al., 2016).

The availability and significant increase in DNA sequence data due to the high 102 sensitivity and accuracy of molecular techniques have attracted the interest of evolutionary 103 104 researchers to identify and characterize previously detected and/or novel species and/or genotypes more accurately. Moreover, studying phylogenetic inferences and epidemiology of 105 106 vector-borne pathogens contributes to the knowledge needed for disease control and 107 prevention. In Egypt, reports for the epidemiology of EVBDs have been limited, except few reports regarding EP (Mahmoud et al., 2016; Elsawy et al., 2021). Therefore, this study aimed 108 to screen Egyptian Equidae and associated arthropods for EVB pathogens such as 109 Piroplasma, Anaplasma, Borrelia, Rickettsia, Coxiella burnetii, and Bartonella using 110 molecular analyses. In addition, equine arthropods were identified using the MALDI-TOF and 111 molecular techniques. 112

113 1. Materials and Methods

114 2.1. Animals and blood sampling

115 This study is a cross-sectional study and included 335 apparently healthy *Equidae* 116 (320 horses and 15 donkeys) using a convenience sampling strategy. The study was approved 117 by the Medical and Veterinary Research Ethics Committee at the National Research Centre, Egypt under the number 19059. Animals were clinically examined, body temperature was measured and inspected for the presence of arthropods on different parts of their body. Blood samples were collected from the 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 for molecular investigations.

123 2.2. Arthropod collection

A total of 166 ectoparasites were obtained from equines (105 sucking flies and 61 ticks) from different localities, as mentioned earlier. All flies and ticks were carefully collected to avoid physical damage. Arthropods from the same animal were counted and gathered in a single tube containing 70% ethanol and transferred to the laboratory for morphological, molecular, and MALDI-TOF MS analyses.

129 2.3. Arthropod identification

130 2.3.1. Morphological identification

Arthropods were identified under the microscope of X56 (Zeiss Axio Zoom.V16,
Zeiss, Marly-le-Roi, France) using taxonomic identification keys (Walker, 2003; EstradaPena et al., 2004; Mullen and Durden, 2009; Onmaz et al., 2013).

134 2.3.2. MALDI-TOF MS identification

135 2.3.2.1. Sample preparation

Ticks and sucking flies that were preserved in 70% ethanol were washed twice in distilled water for 1 min for each time and then dried using sterile filter paper (Kumsa et al., 2016). The legs of the arthropod were cut using a sterile scalpel, put in a sterile 1.5-mL Eppendorf, and incubated at 37°C overnight to evaporate any alcohol residue (Yssouf et al., 2013; Diarra et al., 2017). The rest of the sample was cut longitudinally in two parts; one-half was dissected into small pieces for molecular purpose, and the remaining half was stored at -20°C as backup for any additional studies.

143 2.3.2.2. Sample homogenization and loading on MALDI-TOF target plates

On the cutoff legs, a nip of glass powder (Sigma, Lyon, France) was added into 30 µL 144 145 of a mixture of 70% (v/v) formic acid and 50% (v/v) acetonitrile (Fluka, Buchs, Switzerland; Kumsa et al., 2016). The legs were crushed and homogenized using a TissueLyser II device 146 147 (Qiagen, Hilden, Germany) with 30 movements per second for 1 min and repeated three times (Nebbak et al., 2017). After centrifugation of homogenized arthropod legs (at 2,000 g for 148 30 s), 1 µL of the supernatant of extracted protein was dropped onto a spot of a MALDI-TOF 149 150 polished steel plate (Bruker Daltonics, Wissembourg, France) in quadruplicate (Nebbak et al., 151 2017). At room temperature, the plate was left to dry, and then, each spot was covered with 1 µL of cyano-4-hydroxycinnamic acid (CHCA) matrix solution that was composed of 152 153 saturated α -CHCA (Sigma, Lyon. France), 50% acetonitrile (v/v), 2.5% trifluoroacetic acid (v/v) (Sigma-Aldrich, Dorset, UK), and HPLC grade water (Yssouf et al., 2016). After drying, 154 the plate was loaded into the microflex LT MALDI-TOF MS apparatus (Bruker Daltonics, 155 156 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 157 loading on the MS target plate, matrix quality, and MALDI-TOF apparatus performance 158 159 (Nebbak et al., 2017).

160 2.3.2.3. MALDI-TOF MS parameters

161 The protein mass profiles of each sample were obtained using a microflex LT 162 spectrometer with the flexControl software (Bruker Daltonics) with special parameters 163 recommended by Kumsa et al. (2016). The spectra obtained were visualized by the 164 flexAnalysis 3.3 software and transferred to ClinProTools version v.2.2 and MALDI Biotyper 165 v.3.0 (Bruker Daltonics, Germany) for analysis (Karger et al., 2012).

166 2.3.2.4. Spectrum analysis and reference database creation

For the spectrum analysis, the ClinProTools v2.2 and flexAnalysis v3.3 software programs were applied to evaluate the reproducibility of the MS spectra obtained from the same arthropods' species and assess intraspecies specificity (Fotso Fotso et al., 2014; Nebbak et al., 2016). After the 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 and loaded into the MALDI Biotyper 3.0 to create as
reference spectra (Kumsa et al., 2016; Boucheikhchoukh et al., 2018). As for the upgrade
arthropod MALDI-TOF database, reference spectra were established by spectra at least two
specimens per species of both genders using the algorithm MALDI Biotyper software v3.0
(Bruker Daltonics; Diarra et al., 2017; Boucheikhchoukh et al., 2018).

177 2.3.2.5. 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 corresponded to the degree of similarity between the spectra submitted by the blind test and the MS reference spectra in the database. The identification was considered reliable with an LSV of at least 1.8 (Yssouf et al., 2013; Kumsa et al., 2016). The identification of blind tested samples was assessed by taking the highest LSVs associated with a spectrum quality.

185 *2.4. Molecular techniques*

186 2.4.1. DNA extraction

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

194 2.4.2. Screening of pathogen DNA by quantitative PCR in equines and associated arthropods

Quantitative PCR (qPCR) was performed in all extracted DNA samples (equines and 195 196 associated arthropods) for multi-pathogen DNA screening using genus-specific primers and probes targeting the 5.8S rRNA gene of Piroplasmida (Dahmana et al., 2019), 23S rRNA 197 gene of Anaplasmataceae (Dahmani et al., 2017), gltA gene Rickettsia sp. (Rolain et al., 198 2002), 16S rRNA gene Borrelia sp. (Bottieau et al., 2012), IS1111 intergenic spacer for C. 199 burnetii (Mediannikov et al., 2010), and 16S-23S intergenic spacer for Bartonella sp. (Raoult 200 et al., 2006) (Table 1). The qPCR was applied using the CFX96 Real-Time System (Bio-Rad, 201 202 Marnes-La-Coquette, France). The mixture of qPCR contained 10 µL of Eurogentec Probe PCR MasterMix (Eurogentec, Liège, Belgium), 0.5 µM of primers and FAM-labeled probe, 203 5 µL of DNA template, and 3.5 µL of sterile distilled water to complete the reaction volume 204 to 20 µL. The negative (without any DNA) and positive controls (corresponding pathogen 205 DNA) were added to each reaction to evaluate the reaction. The samples were considered 206 207 positive with the cycle threshold (Ct) lower than 35 Ct (Sokhna et al., 2013).

208 2.4.3. Standard PCR for pathogens identification

209 The positive qPCR samples were subjected to standard PCR and sequencing. For the 210 identification of Piroplasmida (Theileria sp. and Babesia sp.), Anaplasmataceae (Anaplasma sp. and *Ehrlichia* sp.), and *Borrelia* sp., 1,100 bp of the 18S rRNA gene, 520 bp fragment of 211 the 23S rRNA gene, and 1,200 bp of the 16S rRNA gene were used, respectively (Bottieau et 212 al., 2012; Dahmani et al., 2017; Dahmana et al., 2019). The PCR reactions were performed on 213 a thermal cycler (Applied Biosystems, Paris, France) using the AmpliTaq Gold[®] 360 Master 214 Mix (Thermo Fisher Scientific, USA), according to the manufacturer's recommendations. In 215 216 each reaction, negative and positive controls were used. Moreover, 1.5% agarose gel stained with SYBR[®] Safe (Invitrogen, USA) electrophoresis was applied to confirm the amplification 217 and visualized by Image Lab software (Bio-Rad, Marnes-La-Coquette, France). 218

219 2.4.4. Molecular identification of arthropods

Standard PCR was applied to confirm MALDI-TOF identification. Mitochondrial gene (CO1 and 16S rRNA genes) sequencing was used for the identification of flies and tick species through DNA automated thermal cycler (Applied Biosystems, Paris, France) under the same condition as previously described (Table 1) (Chitimia et al., 2010; Abdullah et al., 2016; Boucheikhchoukh et al., 2018). PCR products were purified and sequenced as mentioned earlier (Boucheikhchoukh et al., 2018).

226 2.4.5. Sequencing and phylogenetic analyses

227 The purification of PCR products was applied using NucleoFast 96 PCR plates (Macherey-Nagel, Düren, Germany), according to the manufacturer's recommendations. The 228 229 purified PCR products were sequenced using the BigDye Terminator Cycle Sequencing Kit (3130X1 Genetic Analyzer, ABI PRISM). The sequences obtained were assembled and edited 230 by ChromasPro software (ChromasPro 1.7, Technelysium Pty Ltd., Tewantin, Australia), and 231 232 the corrected sequences were compared with the reference sequences available in GenBank by the BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The obtained sequences of 233 234 Egyptian EVB genotypes and arthropods were recorded in GenBank. These sequences 235 obtained and sequences available in GenBank were aligned using CLUSTAL W in MEGA software X (Kumar et al., 2018). The phylogenetic trees were inferred using the maximum 236 likelihood method and Tamura-Nei model with 1,000 bootstrap replicates in MEGA X 237 software (Tamura and Nei, 1993; Kumar et al., 2018). 238

239 **3. Results**

240 3.1. Morphological identification of arthropods

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). In addition, we examined 15 donkeys and nine horses from Upper Egypt province (Beni Suef) and found 54 *R. annulatus* individuals infesting 15 donkeys and eight horses. Moreover, we found seven *Rhipicephalus* spp. infesting three horses and four donkeys, whose need further identification using the MALDI-TOF technique and molecular biology. The details of collected arthropodsare listed in Table 2.

248 3.2. MALDI-TOF MS analyses of arthropods

For *H. equina*, the random MALDI-TOF test revealed that the obtained spectra did not 249 exist in our database and all tested flies were identical to each other. However, the random 250 251 MALDI-TOF test of *Rhipicephalus* spp. revealed that the obtained spectra were not identical 252 to each other and they did not exist in our database. Therefore, the reference database was essential for the accurate identification of arthropod specimens. After molecular confirmation, 253 H. equina and R. annulatus genotypes from Egypt were introduced as reference MS spectra 254 255 into the laboratory 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 105/105 256 (100%) for *H. equina* $(1.22 \le LSVs \le 2.48, Fig. 2)$ and 61/61 (100%) for *R. annulatus* 257 258 $(1.62 \le \text{LSVs} \le 2.72, \text{Fig. 3}).$

259 *3.3. Molecular identification of arthropods*

Molecular identification was applied on specimens selected for the creation of 260 reference MS spectra. For *H. equina*, based on the CO1 gene, standard PCR and sequencing 261 were applied and succeeded in identifying sucking flies as *H. equina* (GenBank: MK737646) 262 with 99% (589/590) identity to those of *H. equina* from Portugal (GenBank: MN868873). In 263 addition, DNA markers (CO1 and 16S rRNA genes) were used in standard PCR and 264 sequencing on Rhipicephalus spp. specimens selected for the creation of reference MS 265 spectra. The BLAST analysis revealed that R. annulatus was the only Rhipicephalus sp. 266 detected in this study with different genotypes. By the 16S rRNA gene, R. annulatus 267 (GenBank: MK737647 and MK737648) was identified and collected from horses and 268 donkeys in Beni Suef province with 99% (430/434) identity to those of R. annulatus derived 269 from cattle from Beheira province, Egypt (GenBank: KY945491). In further identification 270 through the CO1 gene, the BLAST search confirmed that *Rhipicephalus* sp. was *R. annulatus* 271

272 (GenBank: MZ505561 and MZ505565) with 99% (802/809) similarity to *R. annulatus* from

cattle from the United States (GenBank: KX228534). The phylogenetic position of the five

obtained *R. annulatus* compared with other reference tick species is illustrated in Fig. 4.

275 *3.4. Pathogen detection in arthropods*

All DNA samples of arthropods 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 the DNA of *Anaplasma* and *Borrelia* sp. were detected in *R. annulatus* collected from donkeys and *H. equina* from horses, while *R. annulatus* collected from horses were free from any pathogen DNA (Table 3). All arthropods were found to be free from *Piroplasma*, *Rickettsia*, *C. burnetii*, and *Bartonella* infections.

Anaplasma DNA was detected using qPCR based on the 23S rRNA gene in 21 of 166 282 (12.7%) arthropods, including 27.9% among R. annulatus (17/61) and 3.8% in H. equina 283 (4/105). We were able to amplify and sequence the portion of 23S rRNA gene in nine of the 284 17 samples of R. annulatus collected from donkeys. The BLAST research reported the 285 presence of two different genotype groups that were attributed to two genera of 286 Anaplasmataceae (Anaplasma and Ehrlichia) in R. annulatus. A new A. platys genotype 287 (GenBank: MN614105) was detected in one R. annulatus with 100% (415/415) identity with 288 A. platys-like genotype detected in cattle and sheep from the same locality of Beni Suef 289 province (GenBank: MN626397 and MN626398) and clustered in the same clade (bootstrap 290 value 99, Fig. 5). In addition, this genotype had 99% (409/415) identity with those detected in 291 the blood of New Caledonian dogs (GenBank: KM021425). Another Anaplasma sp. that was 292 detected in R. annulatus was Anaplasma marginale genotype (GenBank: MN614106) with 293 100% (380/380) identity to A. marginale detected in the blood of cattle from Algeria 294 (GenBank: MH321194). Moreover, two Ehrlichia spp. with three different genotypes were 295 found in *R. annulatus*; a new genotype close to *Candidatus* Ehrlichia rustica was derived from 296 one R. annulatus (GenBank: MN614107) with 99% (413/415) identity to those of E. rustica 297

found in Amblyomma variegatum from Côte d'Ivoire (GenBank: KT364330), and Candidatus 298 299 E. rustica genotype was derived from three R. annulatus (GenBank: MN614109) with 100% (415/415) identity to the same reference. Finally, a novel potential Ehrlichia sp. was derived 300 301 from three R. annulatus (GenBank: MN614110) with 98% (404/413) identity with Candidatus Ehrlichia urmitei detected in Rhipicephalus bursa in France. In the phylogenetic tree, the 302 sequence of novel potential Ehrlichia sp. clustered in a well-supported branch (bootstrap 303 304 value 55) with *Ehrlichia ruminantium* (Fig. 5). The phylogenetic position of these genotypes is illustrated in Fig. 5. 305

For *Borrelia* sp., the result of qPCR revealed that 5 of 166 (3%) were positive for *Borrelia* sp., including 3.3% in *R. annulatus* (2/61) and 2.9% (3/105) in *H. equina*. Using 16S rRNA gene sequencing, we identified it as *B. theileri* that derived from two *R. annulatus*. 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 and MN621894). The phylogenetic position of this *B. theileri* genotype is illustrated in Fig. 6.

313 *3.5. Pathogen detection in Equidae*

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

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

Piroplasmida DNA was detected by qPCR in 27 of 335 animals (8.1%, 25 horses and 328 two donkeys). As a result, PCR and sequencing of the 18S rRNA gene portion were 329 performed and allowed the identification of 15 of the 27 qPCR positive samples (4.5%, 13 in 330 horses and two in donkeys). According to the BLAST search, we identified three Piroplasma 331 species: T. equi, a new potential Theileria sp. "Africa," and Theileria ovis. In horses, we 332 333 identified two Piroplasma sp. (T. equi and Theileria sp. "Africa"). By the BLAST research, we obtained two genotypes of T. equi; one genotype was derived from one horse, and another 334 genotype was derived from three horses (GenBank: MN625897 and MN625898) with 100% 335 336 (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" 337 (GenBank: MN625900, MN625901, MN625902, and MN625903) that clustered in a separate 338 clade with a good bootstrap support with the other Theileria sp. "Africa" previously detected 339 in African horses (Fig. 7). In addition, by the BLAST analyses, one genotype from horse was 340 considered to be a new genotype with 99% (917/918) identity to T. equi detected in horse 341 blood from Brazil (GenBank: MG052913). Moreover, three genotypes were identified as T. 342 equi with 100% (923/923) identity to those detected in horse blood from Turkey and Sudan 343 (GenBank: MG569896, MG569893, and AB515309, respectively). In donkeys, T. ovis shared 344 100% (919/919) identity with sheep and buffalo previously detected in the same province in 345 Egypt (GenBank: MN625886 and MN625887). 346

347 **2. Discussion**

348 *Equidae* are used in several beneficial activities for human such as police services, 349 agriculture, and pharmaceutical purposes, in addition to competitive and noncompetitive

leisure pursuits (Payne et al., 2017). Generally, Equidae, especially donkeys, play a 350 351 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 humans (Onmaz et al., 2013). The 352 353 spectrum of EVBDs increased and drew the attention of veterinarians and clinicians to diseases such as piroplasmosis, anaplasmosis, borreliosis, rickettsiosis, bartonellosis, and Q 354 fever (Dantas-Torres et al., 2013). In addition, advances in molecular biology tools and the 355 availability of DNA sequence data facilitate the detection of new pathogen species and even 356 genotypes (Ehounoud et al., 2016). In this study, epidemiological and entomological data on 357 the prevalence of EVBDs infecting Equidae and associated arthropods in two regions of 358 359 Egypt (Capital Cairo and Beni Suef province) were summarized. Moreover, equine arthropod parasites were identified by the MALDI-TOF and molecular techniques. 360

Ixodid ticks Hyalomma and Rhipicephalus and dipteran H. equina are the most 361 362 common arthropods infesting equines (Abdel-Shafy, 1994; Abdel-Shafy, 2000; Onmaz et al., 2013). In this study, traditional taxonomic keys of arthropods revealed the presence of 363 Rhipicephalus sp. as ixodid ticks collected from horses and donkeys of Beni Suef province. In 364 addition, H. equina was morphologically identified that were collected from horses in Cairo. 365 In support of these findings, previous studies found Rhipicephalus sp. (especially R. 366 367 annulatus) in Egypt as the main ixodid ticks infesting cattle and that may infest equines (Abdel-Shafy et al., 2013) and H. equina was louse fly of horses (Hafez et al., 1978). The 368 morphological similarities at both intra- and interspecies levels limit the worth of 369 morphological taxonomic keys such as in Rhipicephalus sp. (Walker et al., 2000; 370 Guglielmone et al., 2014; Nava et al., 2015). Therefore, a combination of the MALDI-TOF 371 MS and DNA markers was applied to identify *H. equina* and *Rhipicephalus* sp. Molecular 372 373 markers (16S rRNA and CO1) revealed one genotype of *H. equina* and five novel genotypes of R. annulatus. Thereafter, the MS spectra of H. equina and R. annulatus were deposed in the 374 database as the reference MS spectra, and then, a blind test was applied on all arthropod 375

samples. Therefore, the combination of molecular identification and MALDI-TOF MS was
successful in the identification of arthropods infesting equines. To our knowledge, *H. equina*and *R. annulatus* were first identified using the combination between MALDI-TOF MS and
molecular identification in Egypt. Later, Elhelw et al. (2021) identified *R. annulatus* from
cattle by a molecular marker in Egypt.

The family Anaplasmataceae includes, among others, two significant genera, 381 Anaplasma and Ehrlichia, which can cause significant infections in a wide range of animal 382 hosts and humans. These infections are mainly transmitted by ticks (Rar and Golovljova, 383 2011). In this study, the overall prevalence of Anaplasmataceae infection in *Rhipicephalus* sp. 384 385 collected from Equidae was 14.7%. This study reports for the first time a novel potential Anaplasma sp. in R. annulatus in Egypt with 1.6% infection rate. This Anaplasma sp. was 386 genetically close to canine A. platys with 99% homology; thus, it was commonly named A. 387 388 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. 5). Furthermore, it forms a separate clade 389 with A. platys-like detected in cattle and sheep from Beni Suef province where R. annulatus 390 was collected from donkeys (Abdullah et al., 2021). To our knowledge, the presence of A. 391 platys-like has never been reported in R. annulatus. Recently, A. platys-like was reported in 392 393 large and small ruminants in Beni Suef, camels in Giza and Cairo (Abdullah et al., 2021), and in cattle from Menoufia province of Egypt (Tumwebaze et al., 2020), implying that this novel 394 potential pathogen circulates in different animal hosts and different provinces of Egypt. 395 Therefore, R. annulatus may act as a vector of this pathogen in Egypt. Moreover, A. platys-396 like was identified in different animal hosts as cattle in Italy (Zobba et al., 2014), Algeria 397 (Dahmani et al., 2015), and Tunisia (Said et al., 2017), camels in Tunisia (Belkahia et al., 398 2015; Selmi et al., 2019), and sheep and goat in South Africa (Berggoetz et al., 2014) and 399 Senegal (Dahmani et al., 2019). In addition, A. marginale was detected in R. annulatus with 400 1.6% infection rate. In Egypt, A. marginale was endemic in different animal hosts such as 401

cattle (Fereig et al., 2017; Al-Hosary et al., 2020; Parvizi et al., 2020), buffaloes (Elhariri et 402 403 al., 2017), and camels (El-Naga and Barghash, 2016). Recently, Al-Hosary et al. (2021) reported A. marginale in R. annulatus. For the genus Ehrlichia, we recorded for the first time 404 405 two different genotypes of Candidatus E. rustica in R. annulatus in Egypt with 6.6% infection rate. One novel genotype of *Candidatus* E. rustica was 99% homology with those of E. 406 rustica found in Amblyomma variegatum from Côte d'Ivoire, and another genotype was 100% 407 408 homology to the same reference (Ehounoud et al., 2016). Moreover, we have identified a new potential Ehrlichia sp. in three R. annulatus with 4.9% infection rate. The sequence of this 409 potential Ehrlichia sp. clustered in a separated clade with E. ruminantium (bootstrap value 410 55). As a result, we had a new potential Ehrlichia sp. and Candidatus E. rustica in R. 411 annulatus that had never been reported before in Egypt. Interestingly, these potential new 412 species were identified in three different regions in the world (France, Côte d'Ivoire, and 413 414 Egypt) and from different tick species (Rhipicephalus, Amblyomma, and Hyalomma sp.) (Ehounoud et al., 2016). Thus, R. annulatus could be a vector for Anaplasmataceae in Egypt, 415 416 and there is a risk of transmission of other novel potential vector-borne diseases. This should 417 be evaluated in future studies.

418 In Africa, most of Borrelia spp. were detected in soft ticks, such as Ornithodoros sp., which is the main vector (Parola and Raoult, 2001). To date, in Africa, Borrelia sp. was 419 identified in hard ticks (Amblyomma and Rhipicephalus sp.) in Ethiopia (Mediannikov et al., 420 2013; Kumsa et al., 2015), Mali (McCoy et al., 2014), Côte d'Ivoire (Ehounoud et al., 2016), 421 Egypt (Hassan et al., 2017), Madagascar (Hagen et al., 2018), and Ecuador (Gioia et al., 422 423 2018). In this study, a new potential B. theileri was identified in two R. annulatus with 3.3% infection rate. The obtained sequence was 99% identical to B. theileri found in Rhipicephalus 424 geigyi in Mali (McCoy et al., 2014). Likewise, the phylogenetic analysis revealed that a new 425 genotype of B. theileri was clustered in the same clade with B. theileri detected in sheep and 426

427 cattle from the same locality of Beni Suef province (Abdullah et al., 2021). In Egypt, *B.*428 *theileri* had previously been detected in *R. annulatus* (Hassan et al., 2017).

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 with that of standard PCR or to the low concentration of pathogenic DNA in fly tissues. Kowal et al. (2016) reported the role of Hippoboscids in the transmission of bacterial pathogens such as *Anaplasma* and *Bartonella*. Moreover, Boucheikhchoukh et al. (2019) detected *Bartonella* and *Wolbachia* sp. in *H*. *equina*.

436 In Equidae blood, we reported Anaplasmataceae DNA in donkeys and Piroplasmida DNA in horses. For Anaplasmataceae, the overall prevalence of anaplasmoses in donkeys 437 was 26.6%, while horses were found free from *Anaplasma* sp. This result was in accordance 438 439 with Ehounoud et al. (2016) who did not find any Anaplasma in horses. A. ovis and A. marginale were the common Anaplasma pathogens of sheep and cattle, respectively 440 441 (Dahmani et al., 2019). However, in our study, we found A. ovis and A. marginale in donkeys. 442 A. ovis shared 100% identity to those of A. ovis in sheep and cattle blood from the same locality of Beni Suef province (Abdullah et al., 2021). Another Anaplasma sp. was A. 443 marginale that shared 100% similarity with those of A. marginale detected in the blood of 444 cattle collected from the same locality of Beni Suef province (Abdullah et al., 2021). A. ovis 445 and A. marginale have never been reported yet in donkeys in Egypt and even Africa. Later, A. 446 reported in donkeys in Pakistan (Asif et al., 447 marginale has been 2019). Therefore, donkeys should be involved in the epidemiology of tick-borne diseases. 448

EP is a protozoan disease caused by *T. equi* and *B. caballi* (Wise et al., 2013;
Mahmoud et al., 2016). 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 may be attributed to self-limiting of *B. caballi* infection and the lifetime persistence of *T*.

equi (Friedhoff and Soule, 1996). In this study, two genotypes of T. equi were pooled in a 453 454 separate clade with T. equi that has already been reported in horses in America (Hall et al., 2013) and Israel (Margalit et al., 2018). Moreover, novel potential Theileria sp. "Africa" 455 genotypes were clustered in a separate clade of a good bootstrap support with the other 456 Theileria sp. "Africa" previously detected in African horses in Senegal and Chad (Fig. 7) 457 (Dahmana et al., 2019). To our knowledge, Theileria sp. "Africa" has never been reported yet 458 459 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 460 Turkey (Ozubek and Aktas, 2018). In the last decade, several researchers have found different 461 piroplasmid species in horses and donkeys and have reduced the host specificity of 462 piroplasmids (Fritz, 2008; Zanet et al., 2017). 463

464 **3.** Conclusion

This study summarized the epidemiological and entomological data of EVBDs 465 infecting equines and associated arthropods in Cairo and Beni Suef province in Egypt. We 466 467 applied, for the first time in Egypt, a combination between the MALDI-TOF and molecular markers for the correct identification of arthropods. In addition, we reported the first detection 468 of A. platys-like, Candidatus E. rustica, and a new Ehrlichia sp. in R. annulatus, A. 469 470 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 471 tick-borne diseases as they serve as reservoirs for these emerging and re-merging pathogens to 472 other animals. 473

474 Ethical Committee approval

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

477 Declaration of Competing Interest

478 The authors declare no conflict of interest.

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487 Authors' contributions

All authors were involved in the design of the study. HHAMA, DA and TKF collected blood and vector samples. HHAMA, DA, TKF, 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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Microorganisms	Targeted SequencePrimers f, r (5'-3') and Probes p (6FAM-		References					
		TAMRA)						
Quantitative PCR primers and probes								
Piroplasmida	5.8S rRNA	f_AYYKTYAGCGRTGGATGTC	Dahmana et al. (2019)					
		r_TCGCAGRAGTCTKCAAGTC						
		p_TTYGCTGCGTCCTTCATCGTTGT						
Anaplasmataceae	23S rRNA (TtAna)	f_TGACAGCGTACCTTTTGCAT	Dahmani et al. (2017)					
		r_TGGAGGACCGAACCTGTTAC						
		p_GGATTAGACCCGAAACCAAG						
Rickettsia sp.	gltA (RKNDO3)	f_GTGAATGAAAGATTACACTATTTAT	Rolain et al. (2002)					
		r_GTATCTTAGCAATCATTCTAATAGC						
		p_CTATTATGCTTGCGGCTGTCGGTTC						
<i>Borrelia</i> sp.	<i>Borrelia</i> sp. Internal transcribed f_GGCTTCGGGTCTACCAC		Bottieau et al. (2012)					
	spacer 16S RNA	r_CCGGGAGGGGGAGTGAAATAG						
	(Bor ITS4)	p_TGCAAAAGGCACGCCATCACC						
Coxiella burnetii Insertion Sequence		f_CAAGAAACGTATCGCTGTGGC	Mediannikov et al. (2010)					
	(IS1111)	r_CACAGAGCCACCGTATGAATC						
		p_CCGAGTTCGAAACAATGAGGGCTG						
Bartonella sp.	Internal transcribed	f_GATGCCGGGGAAGGTTTTC	Raoult et al. (2006)					
	spacer16S	r_GCCTGGGAGGACTTGAACCT						
	(BartoITS3)	p_GCGCGCGCTTGATAAGCGTG						
Standard PCR and	Sequencing primers							
Piroplasmida	18S rRNA	F1- GCGAATGGCTCATTATAACA	Dahmana et al. (2019)					
		F4-CACATCTAAGGAAGGCAGCA						
		F3-GTAGGGTATTGGCCTACCG						
		R3-AGGACTACGACGGTATCTGA						
Anaplasmataceae 23S rRNA		Ana23S-212F-ATAAGCTGCGGGGAATTGTC	Dahmani et al. (2017)					
		Ana23S-753R-TGCAAAAGGTACGCTGTCAC						
<i>Borrelia</i> sp.	16S rRNA	16S-F-GCTGGCAGTGCGTCTTAAGC	Bottieau et al. (2012)					
		16S-R-GCTTCGGGTATCCTCAACTC						
Arthropods	16S rRNA	16Stic.F-CCGGTCTGAACTCAGATCAAGT	Boucheikhchoukh et al.					
Identification		16Stic.R-GCTCAATGATTTTTTAAATTGCTGT	(2018)					
primers	CO1	CO1F-GGAACAATATATTTAATTTTGG	Chitimia et al. (2010)					
		CO1R-ATCTATCCCTACTGTAAATATATG						

Table 1. Primers and probes used for qPCR, standard PCR, and sequencing in this study

Table 2. Information data from the animals studied and their arthropods

Provinces	Gender	Breed	Tick species	Sucking Flies
Animals (Total)	Male/Female	Native/Foreign/Arabic	(Rhipicephalus sp.)	(Hippobosca equina)
			(M/F)	(M/F)
Cairo				
Horses (311)	202/109	271/34/6		105 (70/35)
Beni Suef				
Horses (9)	3/6	9/0/0	0/28	
Donkeys (15)	0/15	15/0/0	4/29	
Total	205/130 (335)	295/34/6 (335)	4/57 (61)	105

Table 3. The prevalence of pathogens in equines and associated arthropods

Animal Hosts /Arthropods	No. of examined Animals/Arthropods	Pathogens amplified	No. of infected Animals/Arthropods (%)
Horse	320	Piroplasmida	13/320 (4.1%)
		Theileria equi	4/320 (1.3%)
		Theileria sp. Africa	9/320 (2.8%)
Donkeys	15	Anaplasmataceae	4/15 (26.6%)
		A. marginale	2/15 (13.3%)
		A. ovis	2/15 (13.3%)
		Piroplasmida	2/15 (13.3%)
		T. ovis	2/15 (13.3%)
R. annulatus	61	Anaplasmataceae	9/61 (14.7%)
		A. platys-like	1/61 (1.6%)
		A. marginale	1/61 (1.6%)
		Candidatus E. rustica	4/61 (6.6%)
		Ehrlichia sp.	3/61 (4.9%)
		<i>Borrelia</i> sp.	2/61 (3.3%)
		B. theileri	2/61 (3.3%)

Figure 1. The arthropods 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 spectrometry profiles of *Hippobosca equina* aligned using flexAnalysis v3.3

software. [Intens] Intensity; [a.u] arbitrary units; [m/z] mass-to-charge ratio. A random color is
used for the spectrum of each specimen

Figure 3. Mass spectrometry profiles of *Rhipicephalus annulatus* genotypes aligned using
flexAnalysis v3.3 software. [Intens] Intensity; [a.u] arbitrary units; [m/z] mass-to-charge ratio. A
random color is used for the spectrum of each specimen

Figure 4. Phylogenetic analysis using the maximum likelihood method based on CO1 for *Rhipicephalus* sp. The new obtained sequences in this study are highlighted (bold)

848 Figure 5. Phylogenetic analysis using the maximum likelihood method based on 23S rRNA for

849 *Anaplasma* sp. and *Ehrlichia* sp. The new obtained sequences in this study are highlighted (**bold**)

850 Figure 6. Phylogenetic analysis using the maximum likelihood method based on 16S rRNA for

851 *Borrelia* sp. The new obtained sequences in this study are highlighted (**bold**)

Figure 7. Phylogenetic analysis using the maximum likelihood method based on 18S rRNA for

853 *Theileria* sp. The new obtained sequences in this study are highlighted (**bold**)



Figure 1.



Figure 2.









Figure 5.





0.02

Figure 7.