Tick-borne Rickettsiales: Molecular tools for the study of an emergent group of pathogens

Tick-borne rickettsiales: molecular tools for the study of an emergent group of pathogens

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Abstract

The use of molecular techniques in recent years has enhanced the sensitivity and specificity of the diagnosis of *Rickettsiales*, a bacterial order which includes significant emerging and re-emerging pathogens of humans and animals. Molecular detection enables the accurate identification at the species level, providing additional information on the epidemiology and course of the clinical cases. Moreover, PCR and enzyme restriction analysis of the vector blood meal can be employed to study the tick feeding source and possibly identify pathogen`s reservoir. Here, we review the molecular tools available for the identification and characterization of tick-borne bacteria from the genera *Rickettsia, Ehrlichia* and *Anaplasma* and for the study of ticks feeding behaviour. We summarize the significant criteria for taxonomic identification of *Rickettsiales* species and propose a procedure algorithm for the classification of bacterial isolates as members of this order.

Key words: *Rickettsia, Ehrlichia*, *Anaplasma,* blood-meal analysis

1. Introduction

The order Rickettsiales is composed of obligate intracellular Gram-negative bacteria infecting eukaryotic cells (Kang et al., 2014). It consists of two families, namely *Rickettsiaceae* and *Anaplasmataceae.* The *Rickettsiaceae* family is integrated by bacteria from the genera *Rickettsia* and *Orientia,* that occupy an intracytoplasmic compartment in the host cell, while the *Anaplasmataceae* family is composed by the genera *Ehrlichia, Anaplasma, Neorickettsia* and *Wolbachia* which occupy an intravacuolar compartment within infected host cells (Dumler et al., 2001). Importantly, *Ehlichia spp.*, *Anaplasma spp.* and several *Rickettsia spp.*, are tick-borne pathogens.

Rickettsia is the most significant genus within the family *Rickettsiaceae.* A modern classification based on whole-genome analysis data (Fuxelious et al., 2007; Gillespie et al., 2007; Gillespie et al., 2008), divides the genus in four groups: the ancestral group (AG), the typhus group (TG), the spotted fever group (SFG), and the transitional group (TRG). AG comprises *R. bellii* and *R. canadensis,* which have no records of pathogenity. TG consists of *R. typhi* and *R. prowazekii.* The SFG is integrated by several species, including *R. rickettsii*, *R. parkeri*, *R. conorii*, *R. massilliae*. Finally, TRG comprises *R. akari*, *R. australis* and *R. felis*. A large number of these organisms are transmitted by hard ticks (*Ixodidae* family) and are associated with emerging life-threatening human zoonoses (Anderson et al., 1992; Buller et al., 1999). Rickettsiae from the TG are not tick-borne, but are transmitted by infected flea, lice and mites (Azad et al., 1988; Durden et al., 2002; Gross, 1996).

The genus *Ehrlichia* belongs to the Anaplasmataceae family and comprises six species (*E. canis, E. chaffeensis, E. muris, E. ruminantium, E. ewingii* and *E. ovis*) from which *E. chaffeensis* and *E. ewingii* are recognized as human zoonotic pathogens (Breitschwerdt et *al.,* 1998; Goldman et *al.,* 1998). Recent reports suggest that some other *Ehrlichia* species might be human pathogens, such as *E. canis,* the etiologic agent of canine monocytic ehrlichiosis (Perez *et al.*, 2006), and organisms closely related to *E. muris*, a rodent and deer associated ehrlichia (Nefedova et al., 2008; Pritt et al., 2012). Finally, *E. ruminantium*-like organisms have been implicated in human infections in South Africa (Louw et al., 2005) and in the USA, where the Panola Mountain Ehrlichia (PME) is reported (Reeves et al., 2007) .

The genus *Anaplasma* (*Anaplasmataceae* family) currently comprises six species: *A. phagocytophilum, A. platys, A. marginale, A. centrale, A. bovis* and *A. ovis* (Dumler et al., 2001). Among these species, *A. phagocytophilum* causes febrile disease in humans and animals and *A. platys* is considered as a low risk pathogen for humans (Otranto et al., 2009), while *A. marginale* is a highly prevalent pathogen of livestock worldwide, causing important losses to production.

Due to the public health implications of bacteria from the order *Rickettsiales*, a proper identification protocol is needed. In this sense, in recent years the number of reports of rickettsiosis and ehrlichiosis has increased (Dumler, 2010; Nicholson et al., 2010). Remarkably, several rstudies in South America revealed the occurrence in new areas (Abarca et al., 2012; André, 2010; Cicuttin and Nava, 2013; García-García et al., 2010; Martinez et al., 2008; Miranda et al., 2011; Miranda et al., 2012; Romer et al., 2014; Tomassone et al., 2008).

Diagnosis using serology faced the difficulty that a detectable level of antibodies against *Rickettsia* sp. does not appear in blood until the day 7 or 10 after the onset of the disease (Leitner *et al*., 2002). In the case of *Ehrlichia* genus, attempts to early identify bacteria by serologic methods, even applying the most widely used techniques, tends to fail in 56–67% of the cases (Childs et al., 1999). For this reason, the diagnosis in the acute phase must rely on the detection of the bacteria in the clinical specimens. Furthermore, direct observation of the *Ehrlichia* morula in stained blood smears also tends to result false negative (22%) (Childs et al., 1999). For *Anaplasma* species, direct observation requires a trained operator and it is useful only for early stages of infection. Similarly, *Rickettsia* species circulate in blood in low numbers even during the acute phase of the disease (Breitschwerdt et al., 1990; Parola et al., 2005). In this sense, the molecular methods become important for diagnosis because, in addition to improving sensitivity, they may give additional information regarding the epidemiology and course of the clinical case.

Up to now, several articles have reviewed the diagnostic methods for the identification of bacteria from the order *Rickettsiales* (Allison and Little et al., 2013; Fenollar and Raoult, 2004; Sparagano et al., 1999). The nowadays popularization of PCR together with the affordable molecular biology consumables due to a wider offer of alternative

kits, give rise to an overwhelming number of diagnostic test reports. In this regard, here we propose an integral revision of the available molecular tools for tick-borne *Rickettsiales* so as to introduce the need to define a core of reliable techniques allowing results comparison among laboratories. Furthermore, we also include a section referring to identification of novel species and the challenge of building up a consensus for taxonomy validation. Finally, we introduce the approaches for the study of ticks feeding behaviour.

2. Tick-borne disease diagnosis based on sample type

When studying tick-borne diseases, we can analyze a diversity of samples. These can be ticks, sampled from the host (feeding ticks) or collected in the environment (questing ticks), or host tissues (blood or other tissues). Samples of various origins can be a source of different information, and contribute to the knowledge of epidemiological aspects regarding the pathogens and their vectors. In example, the detection of the *Rickettsia* spp. DNA in the host blood can generally be achieved only during rickettsemia and is thus an evidence of recent infection (Horta et al., 2007). The finding of a rickettsial agent in a tick can be linked to its reservoir/vector/amplifier host role, or simply to the blood meal that has been taken on an infected vertebrate host. Each result has then to be carefully evaluated and interpreted, according to the known epidemiology of the studied disease.

Different molecular analyses can be performed to detect multiple pathogens in the extracted nucleic acid samples. The detection of co-infections is of crucial medical concern because interactions among pathogens can alter host susceptibility, infection length and clinical symptoms, increasing the disease severity. From an epidemiological point of view, coinfections may modify the infection risk (Andersson et al., 2013; Lommano et al., 2012; Vaumourin et al., 2013). Finally, the extracted DNA can be submitted to a 'blood meal analysis', in order to study the tick feeding behavior and gather information on potential reservoirs of tick-borne agents.

3. Anaplasmataceae family members: identification by means of available molecular tools

3.1 End Point PCR

Many PCR protocols targeting the 16S rRNA gene were developed for the identification of the genera *Ehrlichia*, *Anaplasma* and their species. In Table 1, 16S rRNA gene based protocols for the identification if these genera are summarized. Whatever the protocol used, once the genus is detected (Dawson et al. 1994; Eddlestone et al., 2007; Munderloh et al., 1996; Parola et al., 2000; Wen et al., 2002), subsequent PCRs are required to identify the species of major medical importance. In most cases, the fragments obtained for genus identification can be employed as template for a second step reaction. This is the case of the primers designed for *E. chaffeensis* (Anderson et al.,1992), *E. canis* (Murphy et al., 1998; Wen et al., 1997) and *E. ewingii* (Murphy et al., 1998), that can be used in a second round of amplification employing as template the 479 bp product generated with ECC and ECB oligonucleotides (Dawson et al. 1994). Another nested protocol was proposed by Inokuma et al. (2001a), who designed *Ehrlichia sp.* specific primers and additional *E. muris* and *A. platys* oligonucleotides for a specific second step amplification. For *A. platys* detection, a single step PCR was also proposed by Inokuma et al. (2000).

Finally, Teshale et al. (2015) developed a semi-nested PCR protocol to simultaneously detect the genera *Ehrlichia* and *Anaplasma,* followed by fragment length polymorphism (RFLP) that enables speciation. However, RFLPs protocols are acknowledged as being time-consuming, and exact genotyping cannot always be achieved (Rasmussen, 2012) .

Organism	Primers	Sequence	Amplicon size	Reference
Anaplasma and Ehrlichia	GEPs GEPas	5′CTGGCGGCAAGCYTAACACATGCAAGTCGAACGGA-3′ 5′CTTCTTCTRTRGGTACCGTCATTATCTTCCCYAYTG-3′	432 bp	Eddlestone et al., 2007a
Ehrlichia sp.	EHR16SD EHR16SR	5′-GGTACCYACAGAAGAAGTCC-3′ 5′-TAGCACTCATCGTITACAGC-3′	345 bp	Parola et al., 2000
Ehrlichia sp.	PER1 PER2	5′-TTTATCGCTATTAGATGAGCCTATG -3′ 5′-CTCTACACTAGGAATTCCGCTAT-3′	451 bp	Munderloh et al., 1996
Ehrlichia sp.	Eh-out1 Eh-out2	5′-TTGAGAGTTTGATCCTGGCTCAGAACG-3′ 5′-CACCTCTACACTAGGAATTCCGCTATC-3′	650 bp	Wen et al., 2002
	Eh-gs1 Eh-gs2	5′-GTAATAACTGTATAATCCCTG-3′ 5′-GTACCGTCATTATCTTCCCTA-3′	280 bp	
Ehrlichia sp.	ECC ECB	5′-AGAACGAACGCTGGCGGCAAGC-3′ 5′-CGTATTACCGCGGCTGCTGGCA-3	479 bp	Dawson et al., 1994
E. chaffeensis	HE1 HE3	5′-CAATTGCTTATAACCTTTTGGTTATAAAT-3′ 5′ -TATAGGTACCGTCATTATCTTCCCTAT-3′	389 bp	Anderson et al., 1992
E. ewingii	EE52 HE3	5′-CGAACAATTCCTAAATAGTCTCTGAC-3′ 5′ -TATAGGTACCGTCATTATCTTCCCTAT-3	394 bp	Murphy et al., 1998
E. canis	ECAN HE3	5′- CAATTATTTATAGCCTCTGGCTATAGGA-3′ 5′ -TATAGGTACCGTCATTATCTTCCCTAT-3′	389 bp	
E. canis	ECA HE3	5′ -AACACATGCAAGTCGAACGGA -3′ 5′ -TATAGGTACCGTCATTATCTTCCCTAT-3	410 bp	Wen et al., 1997
Ehrlichia sp.	fD1 EHR16SR	5′-AGAGTTTGATCCTGGCTCAG-3′ 5′-TAGCACTCATCGTITACAGC-3′	760 bp	Inokuma et al., 2001a
E. muris	MURIS GA1UR	5′-GATAGCTACCCATAGCTTTTTTAGC -3′ 5′-GAGTTTGCCGGGACTTCTTCT -3′	414 bp	
A. platys	PLATYS-F PLATYS-R	5′-AAGTCGAACGGATTTTTGTC-3′ 5′-CTTTAACTTACCGAACC-3′	506 bp	
A. platys	PLATYS EHR16SR	5′-GATTTTTGTCGTAGCTTGCTATG-3′ 5′-TAGCACTCATCGTITACAGC-3′	678 bp	Inokuma et al., 2000

Table 1. Primers for *Anaplasmataceae* family identification targeting 16S rRNA gene

In addition to 16S rRNA, other target genes were used for both *Ehrlichia* and *Anaplasma* identification. Dumler et al. (2001) reorganized the genera in *Anaplasmataceae* family based on nucleotide sequences of 16S rRNA and *groEl* genes. Shortly after, Inokuma et al. (2001b) reinforced this new organization employing a well supported *gltA*-based phylogenetic tree. This last gene has been previously validated for identification and phylogenetic analysis of *Rickettsia* (Roux et al., 1997) and *Bartonella* species (Birtles and Raoult, 1996; Joblet et al., 1995), since *gltA* allows a better discrimination among closely related species due to its higher polymorphisms in comparison to 16S rRNA genes. In Table 2, PCR protocols based on *gltA* and *groEl* genes are summarized.

When searching for a defined species in the *Anaplasmataceae* family, a broader range of PCR protocols are available, targeting many different gene fragments. For studying *E.canis,* Stich et al. (2002) developed a nested PCR targeting the *p30* open reading frame (ORF) resulting in a specific reaction that amplifies an exclusive fragment from *E. canis* that is even different from its orthologous *p28* from *E. chaffeensis*. The sensitivity of both the first and the second step were higher than for the nested PCR based on the 16S rRNA gene (100 and 1,000 times respectively). However, only by applying the two steps it was possible to identify *E. canis* carriers in a canine survey (Stich et al., 2002). For studying *E. chaffeensis,* Wagner et al. (2004) amplified a fragment from the *p28* ORF, which is the most studied transmembrane protein family in *E. chaffeensis* (Crocquet-Valdes et al., 2011; Ohashi et al., 2001). The one-step reaction developed on P28 resulted 1,000 times more sensitive than the nested protocol amplifying a 389-pb fragment of the 16S rRNA gene.

For the molecular detection of *E. ruminantium,* diagnostic tests targeting *pCS20* sequences have extensively been used (Faburay et al., 2007; Peter et al., 1995). In the case of *A. marginale, msp5* (major surface protein 5 gene) is the target locus of choice for diagnosis purposes, given that detection of persistently infected cattle is important to control the movement of infected cattle into and from disease-free regions (Torioni de Echaide et al., 1998 ; OIE Terrestrial Manual. 2015). The *ankA* gene is a useful target for *A. phagocytophilum* detection (Beall et. al., 2008). It is an exclusive gene for the *Anaplasma* genus and is also considered one of the major components involved in the pathogenesis of *A. phagocytophilum* (Rikihisa, 2010; Rikihisa, 2011).

Target Gene	Organism	Primers	Sequence	Amplicon size	Reference
gltA	*Anaplasma and Ehrlichia*	F1b EHR-CS779R	5`- GATCATGARCARAATGCTTC-3` 5′- GCNCCMCCATGMGCTCG-3′	119 bp	Inokuma et al., 2002
	E. chaffeensis, E. muris, E.ruminantium, A.phagocytophilum, A. marginale	F4b R1b	5`-CCGGGTTTTATGTCTACTGC-3′ 5`-CGATGACCAAAACCCAT-3′	776 bp	Inokuma et al., 2001c
	E. chaffeensis, E. canis, E. muris, E ruminantium, A.phagocytophilum, A. marginale, A. centrale	EHR-CS136F EHR-CS778R	5-TTYATGTCYACTGCTGCKTG-3 5-GCNCCMCCATGMGCTGG-3	650 bp	Inokuma et al., 2001c
	A. platys	PLA-CSM136F PLA-CS1359R	5′- TTGCAAAAAGTAAGCGGAGC-3′ 5′- AACCACAGGCTTATGACAAC-3′	1495 bp	Inokuma et al., 2002
groEl	*Anaplasma and Ehrlichia*	groEL-643s groEL-1236as	5`-ACT GAT GGT ATG CAR TTT GAY CG-3` 5′-TCT TTR CGT TCY TTM ACY TCA ACTTC-3′	600 bp	Barber et. al., 2010
	E. canis	gro-E.canis163s gro-E.canis573as	5`-AAA TGT AGT TGT AAC GGG TGA ACA G-3′ 5`-AGA TAA TAC CTC ACG CTT CAT AGA CA-3′	410 bp	Otranto et. al., 2010
	A. platys	PLA-HS475F PLAT-HS1198R	5′- AAGGCGAAAGAAGCAGTCTTA-3′ 5′- CATAGTCTGAAGTGGAGGAC-3′	724 bp	Inokuma et al., 2002
	A. platys	GroAplatys-35s GroAplatys-550as	5′- AGCGTAGTCCGATTCTCCAGTTTT-3′ 5′- TCGCCGTTAGCAGAGATGGTAG-3′	516 bp	Beall et. al., 2008
	A.phagocytophilum, A. platys	EphplgroEL(569)F EphplgroEL(1193)R	5′- ATGGTATGCAGTTTGATCGC-3′ 5′- TCTACTCTGTCTTTGCGTTC -3′	624 bp	Alberti et al., 2005
	A. phagocytophilum	EphplgroEL(569)F EphgroEL(1142)R	5′- ATGGTATGCAGTTTGATCGC-3′ 5′- TTGAGTACAGCAACACCACCGGAA -3′	574 bp	
	A. platys	EphplgroEL(569)F EplgroEL(1084)R	5′- ATGGTATGCAGTTTGATCGC-3′ 5′- CATAGTCTGAAGTGGAGGAC-3′	516 bp	

Table 2. Primers based on *gltA* and *groEl* genes for *Anaplasmataceae* family identification

3.2 Real-Time PCR

In recent years, a wide range of real-time protocols (RT-PCR) for the identification of *Anaplasmataceae* species have been developed. They have the advantage of not requiring amplicon sequencing to confirm the positive result and allow the analysis of large sample numbers. Furthermore, they enable quantification of DNA molecules and the reduction of false positive results, since the reaction tubes can remain closed for the interpretation of the results, thus decreasing the possibility of contamination risk.

Available RT-PCR protocols can employ TaqMan or SYBR Green technologies and are focused on diverse genes such as *sodB* (Fe superoxide dismutase), for detection of at least 5 *Ehrlichia spp.* copies per reaction (Qurollo et al., 2014); *dsb* (disulfide bond formation protein gene) for *E. chaffeensis, E. ewingii* and *E. canis* (Doyle et al., 2005); *homp* (hypothetical outer membrane protein gene) (Stoffel et al., 2014) and *msp2* (Courtney et al., 2004; Scorpio et al., 2004) for *E. chaffeensis*, *msp1b* for *A. marginale* (Carelli et al., 2007) and TRP120 for *E. chaffeensis,* which encodes an important immunodominant antigen of this pathogen (Yu et al., 1996; Paddock and Childs, 2003).

In particular, the reaction designed by Bell and Patel (2005) is able to discriminate *A. phagocytophilum, E. chaffeensis* and *E. ewingii,* based on nucleotide differences found in the amplicon through a melting curve. This assay shows a considerably higher sensitivity and specificity when compared to standard PCR protocols (Anderson et al., 1992; Childs et al., 1999; Chu et al., 1998), and allows the simultaneous identification of the three pathogens. The protocol proposed by Sirigireddy et al. in 2005, is a multiplex real-time PCR for the identification of up to five different zoonotic risk bacteria species from the *Anaplasmataceae* family (*E. chaffeensis, E. canis, E. ewingii*, *A. phagocytophilum* and *A. platys*). This molecular tool targets the 16S rRNA gene using oligonucleotides specific for the genus *Ehrlichia* and *Anaplasma,* and implements species-specific TaqMan probes. This assay has an analytical sensitivity of 100 copies of each pathogen and is able to identify coinfections, when the concentration difference between organisms is more than 100 times. In the year 2007, Carelli et al. developed a RT-PCR specific for *A. marginale* which was later improved by Decaro et al. (2008) for the simultaneous detection and quantification of *A. marginale* and *A. centrale* in a duplex

reaction, for pathogenesis studies on bovine acute anaplasmosis. Also, the three main taxonomic loci, *groEl*, 16S rRNA and *gltA,* were employed for RT-PCR protocols in the *Anaplasmataceae* family. Their most important characteristics are summarized in Table 3.

Target Gene	Organism	Primers/ Probes	Discriminatory system	Quantification	Reference
16S rRNA	*E. chaffeensis*	**sense** 5′-AACACATGCAAGTCGAACGG-3′antisense 5′-CCCCCGCAGGGATTATACA-3′	SYBR Green	Yes	Li et al., 2002
	E. chaffeensis	**ECH16S-17** 5′-GCGGCAAGCCTAACACATG-3′ **ECH16S-97** 5′-CCCGTCTGCCACTAACAATTATT-3′ **ECH16S-38** 5′-6-carboxyfluorescein-AGTCGAACGGACAATTGCTTATAACCTTTTGGT-3′	TaqMan probes	Yes	Loftis et al., 2003
	A. platys	**EP-963F:** 5′-CGCAGTTCGGCTGGATCT-3′ **EP-1029R:** 5′-CCCAACATCTCACGACACGA-3′ **EP16Sprobe:** 5′--FAM-TGACGACAGCCATGCAGCACCTG-TAMRA-3′	TaqMan probes	Yes	Eddlestone et al., 2007b
	A.phagocytophilum *A. platys* *E. chaffeensis* *E. ewingii* *E. canis*	**Ehrl/Anapl_F** 5′-CTCAGAACGAACGCTGG-3′ **Ehrl_R** 5′-CATTTCTAATGGCTATTCC -3′ **Anapl_R** 5′-CATTTCTAGTGGCTATCCC -3′ **Ehrl/Anapl_R** 5′-GTATTACCGCGGCTGCTGGCAC -3′ **E. ewi_long.F** 5′CTCAGAACGAACGCTGGCGGCAAGCCTAACACATGCAAGTCGAACGA ACAATTCCTAAATAGTCTCTGACTATTTAGATAGTTGTTAGTGGCAGAC-3′ **A. pla_long.F** 5′CTCAGAACGAACGCTGGCGGCAAGCTTAACACATGCAAGT CGAACGGATTTTTGTCGTAGCTTGCTATGATAAAAATTAGTGGCAGAC-3′ **Ehrl/Anapl_capture** 5′-Biotin-CCCCCCCCCCCCCGGACTTCTTCTRTRGGTACCGTC-3′ **E. chaf** 5′-	TaqMan probes	No	Sirigireddy et al., 2005

		TET/CTTATAACCTTTTGGTTATAAATAATTGTTAG/TAMRA-3′ **E. can** 5′-FAM/TATAGCCTCTGGCTATAGGAAATTGTTAG/TAMRA-3′ **E. ewi** 5′-ROX/CTAAATAGTCTCTGACTATTTAGATAGTTGTTAG/BQH2-3′ **A. pla** 5′-FAM/CGGATTTTTGTCGTAGCTTGCTATGAT/DABCYL-3′ **A. pha** 5′-TET/TTGCTATAAAGAATAATTAGTGGCAGACG/DABCYL-3′ **Ehrl/Anaplcommon** 5′-ROX/TAACACATGCAAGTCGAACGGA/BQH2-3′			
gltA	*A. phagocytophilum*	**ApF:** 5′-**TTTTGGGCGCTGAATACGAT**-3′ **ApR:** 5′-**TCTCGAGGGAATGATCTAATAACGT**-3′ **ApM:** 5′- FAM – **TGCCTGAACAAGTTATG** -3′	TaqMan probes	No	Henningsson et al., 2015
	A. ovis	**sense primer:** 5′-AGGTACCGGGTATCGTTGCA-3′ **anti-sense primer:** 5′-AGGTTTGGATCTGCCTCTGTGA-3′ **probe:** 5′-(FAM)ACATTTACAGGCACACCTCTGGCATGC(BHQ1)-3′	TaqMan probes	Yes	Chi et al., 2015
groEL	*A. centrale*	**AC-For** 5′-CTATACACGCTTGCATCTC-3′ **AC-Rev** 5′-CGCTTTATGATGTTGATGC -3′ **AC-Pb** 5′-TexasRed-ATCATCATTCTTCCCCTTTACCTCGTBHQ2-3′	TaqMan probes	Yes	Decaro et al., 2008
	A. phagocytophilum *E. chaffeensis* *E. ewingii*	**ESp- F** 5′-TACTCAGAGTGCTTCTCAATGT-3′ **ESp-R** 5′-GCATACCATCAGTTTTTTCAAC-3′ **Ec/e-FL** 5′-ATTTCAGCTAATGGAGATAAGAATATAG- FITC-3′ **Aph-FL** 5′-GTCTGCGAATGGAGACAAGAACATAGGA- FITC-3′ **ESp-RD** 5′-Red640-GTAAGATTGCACAGTGTGTTCAAGAAGTCGGTA-Phosphate-3′	Melting curve	No	Bell et al., 2005

Table 3. RT-PCR protocols for *Anaplasmataceae* family identification and quantification

3.3 Tools for studying intraespecific variation at the population level

In order to improve the discrimination power between *E. chaffeensis* populations, Sumner et al. (1999) developed the intraspecific molecular marker VLPT (*Variable-lenght PCR target*). This methodology consists of a nested PCR targeting an ORF encoding a 44-KD polypeptide with four tandem repeats of 90 bp each. In this region, three to six repetitive units can be detected, so that the *E. chaffeensis* populations can be characterized based on the variable length of the obtained amplicons.

Multilocus Sequence Typing (MLST) is a widely accepted method for molecular characterization of bacteria. It enables the genotypic characterization of isolates and the study of the global dispersion of some new variants of pathogens (Mayer et al., 2002). In addition, the data obtained by MLST strategy apply to evolutionary and population studies (Jolley et al., 2000). A MLST scheme has been developed for *E. ruminantium* (Adakal et al., 2009), *A. phagocytophilum* (Huhn et al., 2014) and *A. marginale* (Guillemi et al., 2015). This methodology consists of amplifying and sequencing a set of single copy genes and the further identification of single nucleotides polymorphisms (SNPs) between genotypes.

In Figure 1 we summarize as an algorithm the available molecular protocols for the study of this group of pathogens, together with Rickettsiaceae family (see below).

4. Rickettsiaceae family members: identification by means of available molecular tools

4.1 End point PCR

The serological typing and identification remained the reference diagnostic method for Rickettsiaceae (Philip et al., 1978) until Roux et al. (1997) analyzed and compared citrate synthase (*gltA*) sequences from different *Rickettsia* species. They also compared *gltA* with 16S rDNA sequences and found that the average rate of sequence change in *gltA* was faster than in 16S rRNA, which indicates that *gltA* was less conserved in rickettsiae. This result would suggest that for discrimination within the *Rickettsia* genus, *gltA* sequences may be quite valuable. In the same publication, the authors also suggested two other molecular candidates: genes encoding rOmpA and rOmpB

proteins. These molecules are specific for the SFG rickettsiae and the members of the *Rickettsia* genus respectively, and have been shown to exhibit high degrees of difference between species employing PCR-RFLP (Eremeeva et al., 1994; Regnery et al., 1991; Roux et al., 1996) or sequencing (Roux et al., 1996) than other molecules (gltA, 17-kDa protein, 16S rRNA). Accordingly, Fournier et al. (1998) amplified the whole *ompA* gene (excluding the central region containing the tandem repeat units) of 21 SFG rickettsiae isolates and confirmed that the intra-SFG variability in *ompA* was markedly higher than that of 16S rRNA or *gltA* genes. Later, by amplifying and sequencing the *ompB* gene of 24 representatives of the *Rickettsia* genus, Roux and Raoult (2000) observed that the study of this protein, which has common epitopes to both SFG and TG rickettsiae, required to be analyzed in combination with *gltA* and *ompA* to obtain a reliable phylogeny for all the bacteria in the *Rickettsia* genus.

Since these genes resulted to have a high discrimination power, many PCR protocols have been described using them as targets (Table 4). Even, Regnery et al. (1991) developed an identification and species discrimination system, based on the amplification of two fragments from the *gltA* gene, followed by an endonuclease restriction of the second amplicon. Through this PCR scheme followed by RFLP, *Rickettsia* species and genotypes can be differentiated. Kidd et al. (2008) developed a PCR with primers targeting a small region of the *ompA* gene for the identification of SFG rickettsiae. The sensitivity of this reaction was improved (up to 5 DNA copies) when the primers were adapted to a real-time PCR (see below). This PCR protocol appeared to be more sensitive than the assay amplifying the largest portion of the *ompA* gene described by Roux et al. (1996), and has enough variability in this smaller region to distinguish species based on sequencing. A protocol targeting the 17-kD antigen gene, that implies a nested PCR followed by RFLP analysis, was designed for *R. conorii.* Although this technique is not specific for this species (since the nested PCR also amplifies DNA from *R. australis*, *R. rickettsii*, *R. akari*, *R. typhi,* and *R. prowazekii*), it enables differentiation of SFG from TG rickettsiae. For this purpose, a RFLP test based on the digestion of the 214-bp PCR product with the restriction enzyme *Bfa*I is necessary. The SFG rickettsiae amplicon contains one *Bfa*I site, resulting in two

fragments, while the TG rickettsiae amplicon contains two sites, which generate three fragments (Leitner et al., 2002).

Fournier and Raoult (2004) developed a suicide PCR for the detection of both TG and SFG rickettsiae from skin biopsy specimens. A suicide PCR consists of single-use primers; in this case oligonucleotides were selected from conserved regions flanking 300 to 600-bp variable fragments of genes present in both *R. conori* and *R. prowazekii* genomes (targeting, each year of the study, a new gene that had never been amplified before in the laboratory). The positive PCR products were sequenced in both directions. This technique, based on single-use primers targeting single-use DNA fragments, presents a specificity of 100% and a sensitivity of 68%, but is 1.5 times more sensitive than the conventional PCR by Regnery et al. (1991). The authors suggest that suicide PCR should be reserved for use on patients suspected of having a rickettsiosis but with negative regular PCR results.

Rickettsia group	**Primers sequence**	**Target Gene**	**Amplicon size**	**Reference**
Rickettsia spp.	**120-M59:** 5′- CCGCAGGGTTGGTAACTGC-3′ **120–807:** 5′- CCTTTTAGATTACCGCCTAA-3′	*ompB*	833 bp	Roux and Rault, 2000
Rickettsia spp.	**CS-78** 5′- GCAAGTATCGGTGAGGATGTAAT-3′ **CS-323** 5′-GCTTCCTTAAAATTCAATAAATCAGGAT-3′	*gltA*	401 bp	Labruna et al., 2004
Rickettsia spp.	**RpCS.415** 5′-GCTATTATGCTTGCGGCTGT-3′ **RpCS.1220** 5′- TGCATTTCTTTCCATTGTGC-3′	*gltA*	806 bp	de Sousa et al., 2006
Rickettsia spp.	**RCK/23-5-F:** 5′-GATAGGTCRGRTGTGGAAGCAC-3′ **RCK/23-5-R:** 5′-TCGGGAYGGGATCGTGTGTTTC-3′	*23S-5S*	388 bp	Jado et al., 2006
SFG *Rickettsiae*	**RpCS.877p** 5′- GGGGGCCTGCTCACGGCGG-3′ **RpCS.1258n** 5′- ATTGCAAAAAGTACAGTGAACA-3′	*gltA*	381 bp	Regnery et al. 1991
	Rrl9O.70p 5′- ATGGCGAATATTTCTCCAAAA-3′ **Rrl9O.602n** 5′- AGTGCAGCATTCGCTCCCCCT-3′	*ompA*	532 bp	
SFG *Rickettsiae*	**Rr 190.70p** 5′- ATGGCGAATATTTCTCCAAAA -3′ **190-701** 5′- GTTCCGTTAATGGCAGCATCT-3′	*ompA*	631 bp	Roux et al., 1996
SFG *Rickettsiae*	**SLO1F:** 5′- CACCACCTCAACCGCAG-3′ **SLO1R:** 5′- GCCGGGGCTGCAGATTG-3′	*ompA*	489 bp	Raoult et al., 2002
SFG *Rickettsiae*	**107F** 5′- GCTTTATTCACCACCTCAAC-3′ **299R** 5′- TRATCACCACCGTAAGTAAAT-3′	*ompA*	209-212 bp	Kidd et al., 2008
SFG *Rickettsiae*	**Rc.rompB.4.362p:** 5′-GTCAGCGTTACTTCTTCGATGC -3′ **Rc.rompB.4.836n:** 5′-CCGTACTCCATCTTAGCATCAG-3′	*ompB*	475 bp	Choi et al., 2005
	Rc.rompB.4.496p: 5′-CCAATGGCAGGACTTAGCTACT-3′ **Rc.rompB.4.762n:** 5′-AGGCTGGCTGATACACGGAGTAA-3′		267 bp	

Table 4. Primers for identifying SFG *Rickettsiae*

For species-specific identification, few PCR protocols are available (Roult et al., 2001; Tzianabos et al., 1989). In an attempt to increase the sensitivity and avoid false positive amplifications, Raoult et al. (2001) developed a suicide PCR. This scheme targets the *ompA* gene from *R. africae* and is based on three sets of two pairs of single-use primers that amplify non-overlapping fragments in a nested protocol. The main disadvantage of the suicide PCR is that once a pair of primers is used in a laboratory, it can not be re-used.

4.2 Real-Time PCR

Because of the intracellular habitat of *Rickettsiae* and their slow generation time, the use of traditional techniques to quantify the number of viable Rickettsiae in a sample is laborious, inaccurate, and tedious (Eremeeva et al., 2003). On the other hand, RT-PCR has significant advantages over end-point PCR methods. For instance, RT-PCR requires shorter assay times; it does not require gel electrophoresis and has the capacity of analyzing large numbers of samples with fully automated equipment. Moreover, RT-PCR can be used as a quantitative method and for the identification of *Rickettsia* species on samples contaminated with other microorganisms.

As it was mentioned above for *Anaplasmataceae*, RT-PCR protocols can employ TaqMan or SYBR Green chemistries and are focused on diverse genes. In the case of *Rickettsiaceae* family, the genes usually targeted are: *gltA, ompA* and *ompB* (Table 5).

Organism	Target Gene	Primers/ Probes	Discriminatory sistema	Quantitative	Reference
Rickettsia spp.	*gltA*	**CS-5** 5′-GAGAGAAAATTATATCCAAATGTTGAT -3′ **CS-6** 5′-AGGGTCTTCGTGCATTTCTT -3′ **probe** 5′- 6-FAM dCATTGTGCCATCCAGCCTACGGT BHQ1-3′	Fluorogenic probe	No	Labruna et al., 2004
Rickettsia spp.	*gltA*	**gltAF** 5′-TCGCAAATGTTCACGGTACTTT-3′ **gltAR** 5′-TCGTGCATTTCTTTCCATTGTG-3′	Melting curve (SYBR Green)	No	Stenos et al., 2005
	ompB	**ompBF** 5′-CGACGTTAACGGTTTCTCATTCT-3′ **ompBR** 5′-ACCGGTTTCTTTGTAGTTTTCGTC-3′		No	Paris et al., 2008
SFG	*ompA*	**RR190.547F** 5′-CCTGCCGATAATTATACAGGTTTA -3′ **RR190.701R** 5′-GTTCCGTTAATGGCAGCAT -3′	SYBR Green	Yes	Eremeeva et al., 2003
R. felis *R. conorii* *R. typhi*	*gltA*	**CS877F** 5′-GGGGGCCTGCTCACGGCGG-3′ **CS1258R** 5′-ATTGCAAAAAGTACAGTGAACA-3′	Melting curve (SYBR Green)	Yes	Rolain et al., 2002

Table 5. RT-PCR protocols for *Rickettsiaceae* family identification

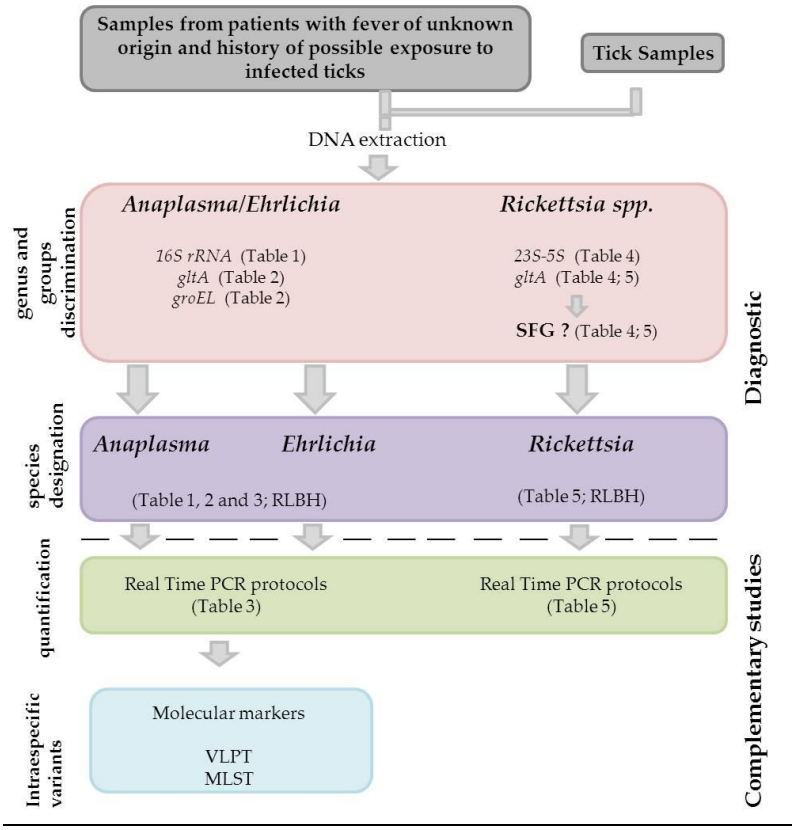

Figure 1. Suggested algorithms for Rickettsiales identification and characterization.

5 Middle and High-throughput tools for the study of Rickettsiales

In addition to the molecular protocols described for the identification of one or a group of pathogens, dedicated tools for the simultaneous identification of this group of pathogens have been developed. In this sense, the Reverse Line Blot Hybridization (RLBH), DNA microarray and next generation sequencing techniques can be mentioned.

The RLBH methodology enables the simultaneous detection of different bacterial species from diverse samples. It consists on a PCR assay (nested or unique) that is then coupled to a hybridization reaction. Each species can be identified by a species-specific oligonucleotide that acts as a probe and that it is covalently bound to a membrane where the hybridization reaction, with PCR product, will take place. The name of the method arises from the reversed arrangement of the components. A device called "line blotter" is required to carry out this methodology, and enables the evaluation of different samples applying different probes that are immobilized in a transverse sense

to the line where the amplification product will be loaded. Furthermore, the oligonucleotides employed in the amplification step are biotinilated in the 5`terminus giving rise to a labelled PCR product, which enables the hybridization signal recognition through a coupled detection reaction using streptavidin-peroxidase, followed by a chemiluminescence developed reaction, visualized by exposing the membrane to X-ray film. The membrane with the specific oligonucleotides in different lines is placed in a transversal sense in relation to that in which the PCR products are loaded, so that the different species simultaneously amplified by PCR can be specifically detected in the intersection of the oligonucleotide and the PCR product lines. This membrane can be re-used up to 20 times and has the major advantage of enabling the simultaneous analysis of up to 43 samples in the same membrane (Kong and Gilbert; 2006). RLBH membranes have been developed to identify pathogens from the *Anaplasmataceae* family (Bekker et al., 2002; Tomassone et al., 2008). The PCR step employs oligonucleotides which target a conserved region of the 16S rRNA of *Anaplasma* and *Ehrlichia*, containing an hypervariable region with polymorphisms specific for each of the known species in the two genera (Bekker et al., 2002). After amplification, the product is loaded in the membrane including specific oligonucleotides for *A. phagocytophilum*, *A. marginale*, *A. centrale*, *A. ovis*, *E. chaffeensis, E. ruminatium*, *Ehrlichia. sp. omatjenne* and *E.canis*. Generic and species-specific probes were also designed for eleven *Rickettsia* species by Jado et al. (2006). The PCR required in the first step targets the 23S-5S internal transcribed spacer (ISR), and the resulting amplicons are then hybridized to a membrane containing the generic (*Rickettsia* catch-all, SFG and TG) and species-specific probes (*R. akari, R. bellii, R. slovaca, R. conorii, R. aeschlimannii, R. rickettsii-R. sibirica, R. helvetica, R. felis, R.australis, R. prowazekii, R. typhi*). For *R. rickettsii* and *R. sibirica*, a common probe was designed due to the high homology of their 23S-5S ISR sequences, and amplicon sequencing is needed for discriminating between the two species. RLBH is a useful tool for diagnostic and epidemiologic surveys and enables the detection of new microbial species. Although being a simple methodology of easy interpretation, it is specific enough to detect single-base-pair changes and showed high sensitivity for Rickettsiales identification (Schouls et al., 1999). Jado et al. (2006) report a sensitivity of one plasmid copy per

reaction, which was determined in the presence of foreign DNAs from different clinical and environmental samples. Moreover, RLBH membranes can be designed combining probes for different pathogens. In this sense, specific genus and species probes for *Rickettsiaceae* and *Anaplasmataceae* can be placed in the same membrane so that simultaneous detection of bacteria from the order can be carried out in the same reaction.

The DNA microarray is another tool based on molecular hybridization. El-Ashker et al. (2015) have developed a DNA microarray chip for the simultaneous identification of cattle tick-borne pathogens including *Anaplasma* species. As it was described for the RLBH, after a first step of amplification of a 16S rRNA biotinylated amplicon, the PCR fragments are then hybridizated and incubated with streptavidin-peroxidase conjugate for further visualization through a Chip Scanner. Nevertheless, the validation of this microarray is still in progress and has not been widely adopted.

The molecular approaches described above provide a way to gauge diversity in a microbial community, but considering that it is likely that ticks are host to a larger diversity of, as yet non-described, microorganisms, those molecular tools are not an efficient way to determine the presence or absence of any microbes in a sample, nor they provide robust information on relative abundances of a given microbe. In this sense, the advent of next generation sequencing techniques (NGS) promises to surmount many of the limitations in current molecular approaches to microbial diversity studies (Clay and Fuqua, 2010).

6. Suggested algorithms for taxonomic characterization and identification of novel species

Classification of members of the genus *Rickettsia* was based on mouse serotyping (Philip et al., 1978), Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) or pulsed-field gel electrophoresis (PFGE) (Fournier et al., 2003) until Fournier et al. (2003) established objective guidelines for isolate classification based on the sequencing of specific genes. These guidelines enable the classification of an isolate as member or not of this genus at various taxonomic levels. Previously, DNA taxonomy has been demonstrated to be useful for bacterial classification purposes (Bouyer et al.,

2001; Niebylski et al., 1997; Tautz et al., 2003). The complete published sequences of the 16S rRNA (*rrs*), *glt*A, *ompB* and *gene D* (encoding the surface-exposed high-molecular-weight protein PS120), and the 5´end of the *ompA* gene of all validated *Rickettsia* species (except for *R. peacockii*) were compared. Then, based on the percentage of homology of each gene in relation to the known *Rickettsiae*, the unknown bacteria can be identified at the different taxonomic levels (Fournier et al., 2003).

In the last few years there have been reports of isolates that were characterized as *Ehrlichia spp*. by molecular methods, but failed to assign a species due to scarce sequence similarities against validated *Ehrlichia* species. In this regard, a novel tick-transmitted *Ehrlichia* called Panola Mountain was reported based on phylogenetic reconstruction from fragments of the 16S rRNA, *gltA, map1, map2*, and *ribonuclease III* genes. This organism was first detected in a goat and *A. americanum* ticks from a park in the metropolitan area of Atlanta, GA, U.S.A (Loftis et al., 2006). On the other hand, *Ehrlichia sp. AvBat* was proposed as a novel species by Socolovschi et al. (2010) after analyzing phylogenetic trees based on the *gltA* and 16S rRNA genes. Later, using a different set of target regions, Cruz et al. (2012) and O´Nion et al. (2015) reported the isolation of potentially novel *Ehrlichial* species from the hemolymph of *Rhipicephalus microplus* engorged females that had been collected from naturally infested cattle in Brazil and from horses in Nicaragua, respectively. Even, *Candidatus* status genera were proposed after phylogenetic reconstruction of diverse gene sequences, like"*Candidatus* Xenohaliotis" (Friedman et al., 2000) and "*Candidatus* Neoehrlichia" (Schouls et al., 1999).

This findings remark the need of a clear and agreed algorithm for new *Ehrlichia* species identification, similar to the one available for *Rickettsia* genus (Fournier et al., 2003).

A useful tool for the identification of new species of both *Anaplasmataceae* and *Rickettsiaceae* is the RLBH method. As a first step, a conserved region can be amplified by PCR, the targets are the 23S-5S internal transcribed spacer for *Rickettsia* genus, and the 16S rRNA gene for *Ehrlichia* and *Anaplasma* genera. Then by applying RLBH, the hypervariable region can be evaluated, since the membrane contains specific probes with polymorphisms for each of the known species of the three genera. If analyzing a sample, the common fragment is detected but no species-specific probes are identified,

evaluation of possible new species should be considered through different target genes. For *Ehrlichia* and *Anaplasma* genera, three target genes are suggested (Dumler et al., 2001; Inokuma et al., 2001) while for *Rickettsia spp.* a scheme of five target genes is proposed (Fournier et al., 2003) (Figure 2).

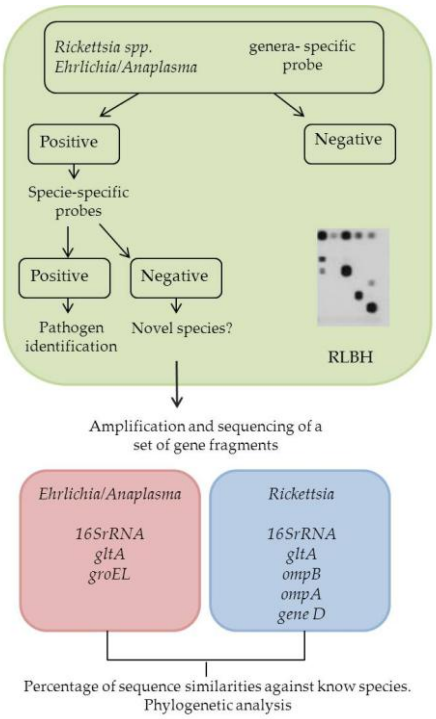

Figure 2. Suggested algorithms for taxonomic characterization and identification of novel *Rickettsia, Anaplasma* and *Ehrlichia* species

7. Vertebrate reservoir host identification through blood meal analysis

Understanding the dynamics of pathogen transmission from infected wild hosts is critical for the study of tick-borne-pathogen epidemiology. Ticks can acquire pathogens by two main transmission ways: vertical (transovarial), in which the pathogen is transmitted from the female to offspring during egg development; and horizontal, in which pathogens are acquired during the blood meal feeding from reservoir or infected host (Allan et al., 2010). Moreover, transmission can occur between infected and susceptible ticks feeding simultaneously in close proximity on the same host (cofeeding transmission); such mechanism may enable the transmission of a pathogen even between ticks feeding on a non-competent host (Randolph et al., 1996). Efforts to identify reservoirs have historically been laborious, requiring in many cases the

capture of potential hosts, their experimental infection with the organism of interest and the subsequent evaluation of their efficiency in the transmission process under controlled conditions (xenodiagnosis; LoGiudice et al., 2003). Even with such complex procedures, it is not always possible to assess the true importance of each vertebrate in the pathogens maintenance (Brisson et al., 2008). Xenodiagnostic procedures are essential to confirm the capacity of an infected vertebrate to transmit the pathogen to the vector, and of being infected by a vector. However, the molecular identification of the host DNA in the arthropod blood meal, together with the pathogen detection in the tick, may be a valid tool for the identification of possible reservoir sources of blood pathogens. This is particularly valuable in the study of the epidemiology of pathogens whose transmission cycles are not completely known, as in the case of several Rickettsiales. Unfortunately, diverse factors can interfere with the detection of vertebrates DNA in vector samples. Among such factors, we have the scarce host blood volume available in the tick (due to degradation or the incapacity of the tick to engorge until repletion on certain hosts). The choice of the genome region that will be the target for molecular detection is also critical (Kent, 2009), since degradation of host DNA occurs after a period of time and only short DNA fragments are found.

Molecular protocols for detection of host DNA are in constant development, with the use of new targets and primers (Wodecka et al., 2014). One available tool is DNA sequencing of amplicons obtained from vertebrate conserved genes like the mitochondrial cytochrome b (CytB) (Pierce et al., 2009; Che Lah et al., 2015) or vertebrate rRNA genes. This option is accurate and specific but expensive when a great amount of samples are analysed (Kent, 2009); moreover, it is not always possible to identify multiple blood meals within a vector. An alternative is the use of PCR-RFLP (Kirsten and Gray, 1996; Wodecka et al., 2014), which employs restriction endonucleases enzymes for the fragmentation of amplicons obtained from vertebrate conserved genes. This digestion results in differentially sized segments which are resolved visually by gel electrophoresis. The disadvantage of this technique is that, for a correct identification, a library of restriction patterns from known vertebrates is required (Kent, 2009). Another available tool is the PCR amplification of vertebrate DNA with universal primers, followed by RLBH; 18S rRNA gene (Allan et al., 2010;

Pichon et al., 2003; Pichon et al., 2005) and 12S rDNA gene (Humair et al, 2007) are generally used as targets. Recently, a new sensitive method for tick blood meal analysis has been reported: a non-targeted, genome-free, proteomics-based, spectral-matching methodology (Önder et al., 2013). This tool employs direct spectral matching to determine the vertebrate species from which the tick acquired the blood. A great advantage is that heavily degraded DNA can be detected, even when several months from the last meal have passed. Accordingly, further advances in the field are expected with the development of next-generation sequencing (NGS). This technique may enable the simultaneous identification of the vertebrate blood and pathogen infection within the arthropod vectors; in addition, it may allow the identification of multiple blood meals and requires a small amount of vertebrate reservoir DNA. The standardization of the methodologies and a decrease in costs are needed to make NGS a commonly used tool for the analysis of blood meal.

8. Conclusions

Rickettsiosis, ehrlichiosis, and anaplasmosis are increasingly reported in humans and animals worldwide, and tools for their recognition and prevention are needed. Although several techniques have been developed in the recent years, there is a need for a reorganization of the diagnostic procedures to make them more uniform across laboratories and geographic areas.

In our experience, the approach of choice should start with the identification of pathogens from *Rickettsiaceae* and *Anaplasmataceae* families at the genus level in host and tick samples, employing the main target genes validated for each family. Then, for positive reactions, further analysis can be carried out. For this purpose, groups and species can be characterized by sequencing the amplified products or applying specie-specific real-time or end point PCR protocols. For *Ehrlichia* and *Anaplasma* species, isolate characterization through specific molecular markers is also feasible. These methodologies are useful for molecular epidemiology surveys as they enable genotypic characterization of isolates and the study of the origin of new variants of pathogens.

Although NGS is not widely used for diagnostic purpose so far (Lefterova et al., 2015), it is promising for microbial diversity studies, including the study of the tick

microbiome. Moreover, the same tick sample examined for pathogen identification can provide data for the study of the tick feeding behaviour, through the blood meal analysis. This approach is critical for understanding tick-borne-diseases as it brings information related to vertebrates that may act as reservoir or amplifier hosts (Labruna et al., 2009), contributing to the dispersion of tick-borne pathogens. The identification of new reservoir/amplifier hosts is a challenge in a constantly changing environment, where the increasing contact rate between wild and domestic animals, and animals and human beings, leads to the emergence and re-emergence of infectious diseases.

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