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Guidelines for the Detection of *Babesia* and *Theileria* Parasites

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Abstract

The genera *Babesia* and *Theileria* (phylum Apicomplexa, order Piroplasmida) are mainly transmitted by Ixodid ticks in which the sexual part of their life cycle followed by sporogony takes place. They include protozoan parasites that infect erythrocytes of a variety of vertebrate hosts, including domestic and wild animals, with some *Babesia* spp. also infecting humans. *Babesia* sporozoites transmitted in the tick's saliva during the bloodmeal directly infect erythrocytes, where they asexually multiply to produce pear-shaped merozoites in the process of merogony; whereas a pre-erythrocytic schizogonic life stage in leukocytes is found in *Theileria* and precedes merogony in the erythrocytes. The wide spectrum of *Babesia* and *Theileria* species and their dissimilar characteristics with relation to disease severity, transmission, epidemiology, and drug susceptibility stress the importance of accurate detection of babesiosis and theileriosis and their causative agents. These guidelines review the main methods currently used for the detection of *Babesia* and *Theileria* spp. for diagnostic purposes as well as epidemiological studies involving their vertebrate hosts and arthropod vectors. Serological methods were not included once they did not indicate current infection but rather exposure.

Keywords: *Babesia*, diagnosis, in vitro culture, PCR, *Theileria*

Introduction

The genera *Babesia* and *Theileria* (phylum Apicomplexa, order Piroplasmida) are protozoan parasites that infect erythrocytes of a variety of vertebrate hosts, including domestic and wild animals, with some *Babesia* spp. also infecting humans. The parasites are transmitted by Ixodid ticks. In the arachnid host, sexual reproduction takes place followed by sporogony. Resulting sporozoites are transmitted in the tick's saliva during the bloodmeal. *Babesia* spp directly infect erythrocytes, where they asexually multiply to produce pear-shaped merozoites in the process of merogony. In contrast, *Theileria* spp undergo a pre-erythrocytic schizogonic life stage in leukocytes that precedes merogony in the erythrocytes (Uilenberg 2006, Hunfeld et al. 2008).

Babesia species have traditionally been categorized according to their vertebrate hosts and morphology into large ($\sim$3–5 μm) and small forms (0.5–2.5 μm) based on the size of their merozoites when viewed by light microscopy in stained blood smears. More recent molecular studies involving gene sequencing and phylogenetic analyses have uncovered a much broader diversity among *Babesia* species, even those of a similar size and infecting the same animal host. Such studies have shown that babesial species actually group into several discrete clades that separate what appear to be identical parasites by light microscopy, thus enforcing the importance of the use of molecular techniques for species identification (Criado-Fornelio et al. 2004, Schnittger et al. 2012, Yabsley and Shock 2012). In the past decade, several new pathogenic species of *Theileria* have been identified and

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pathogenic strains of other previously low-pathogenic species have emerged (Morrison 2015).

Clinical babesiosis and theileriosis are usually associated with anemia due to erythrocyte destruction and hemolysis, with lymph node enlargement characteristic of theileriosis. Infected hosts may suffer from acute onset of fever and lethargy, and the disease may progress to severe fatal organ failure. On the other hand, animals may become chronic carriers of infection, often without manifesting clinical abnormalities. *Babesia* species possess variable degrees of virulence, with some species able to cause severe disease whereas others only induce mild-to-moderate illness. Different species are transmitted by different tick vectors, and they may, therefore, be limited geographically to the regions where their vectors are prevalent. Furthermore, drug susceptibility differs among species and especially between the small and large forms of parasites (Solano-Gallego and Baneth 2011, Mosqueda et al. 2012).

The wide spectrum of *Babesia* and *Theileria* species and their species-specific characteristics with relation to disease severity, transmission, epidemiology, and drug susceptibility underline the importance of accurate identification of the causative agents. These guidelines review the main methods currently used for the detection of *Babesia* and *Theileria* spp. for diagnostic purposes as well as epidemiological studies involving their vertebrate hosts and arthropod vectors. Serological methods were not included, because they indicate exposure, rather than current infection and often lack species specificity.

Microscopic Examination

Detection of Babesia *in blood smears*

Sensitivity and specificity of parasite identification based on microscopical examination of blood smears are dependent on the experience and proficiency of the examiner. Nevertheless, this technique is a cheap and easy method that is readily available in all standard laboratories and possibly even in the field for the detection of acute cases. Moreover, knowledge of the endemic species in an area and differentiation between ''large'' and ''small'' *Babesia* species can help to narrow down the identity of the detected piroplasms (Table 1). Most commonly, thin blood smears are air-dried, fixed (absolute methanol, 2 min), and stained in a 1:20 dilution of Giemsa stain (40 min). Other stains such as Romanowsky, Diff-Quik, and Wright's can also be used. Sensitivity may be improved by using ''thick'' blood smears. However, identification of the parasite outside the host cell and in the presence of the many artefacts produced by this method is challenging. Species that accumulate in capillaries and tissues, such as *Babesia bovis*, are more readily detected in capillary blood (collected from the ear tip or nail bed) or in crush smears of certain organs such as brain (gray matter of the cerebral cortex), spleen, liver, or kidneys (Figueroa et al. 2010). All other species are best diagnosed from anticoagulated venous blood.

With regard to human infections, in areas where malaria is abundant, differentiation between *Babesia* and *Plasmodium* spp. can be challenging. The main differences are that *Babesia*-infected erythrocytes lack hemozoin deposits (although these are not always present in infections with young *Plasmodium* trophozoites either) and occasionally present tetrade or ''Maltese cross'' formations, which are never observed in *Plasmodium* spp. infection. In contrast, the latter may exhibit distinctive schizonts and gametocytes (Kjemtrup and Conrad 2000).

Detection of Theileria *in blood and tissue samples*

The same protocols are used to fix and stain *Theileria* parasites in thin blood/lymph node or spleen smears. In erythrocytes, *Theileria* merozoites are predominantly rod shaped and up to 2.0 μm long and 1.0 μm wide. Round, oval, and ring-shaped forms also occur. Multiple parasites per erythrocyte are common. In the cytoplasm of lymphocytes, two types of schizonts (Koch's blue bodies) can be found: macroschizonts and microschizonts, both about 8.0 μm, containing up to 8 and 36 small nuclei, respectively (Urquhart et al. 1996).

Microscopic detection in ticks

Although sporozoites of both *Babesia* and *Theileria* species can be detected in whole tick salivary glands (SG) using the Feulgen technique or in SG histological sections, after staining with basic dye, they can only be differentiated by polymerase chain reaction (PCR). However, it provides a quick and easy method for screening a pasture before introducing cattle onto it.

For this purpose, unfed or 4–5 day engorged ticks are embedded in a small Petri dish in a 1 cm-diameter circle of melted paraffin, with the tick's dorsal surface facing up. To lift the scutum, an incision is made with a scalpel blade around the margin of the body, starting and ending at the base of the capitulum. This facilitates removal of the gut and exposure of the SG (Edwards et al. 2009). Trachea fragments are removed, and the whole SG are immersed in physiological saline solution. SG are then fixed for 15–30 min in Carnoy's fluid (Marx and Stern 2003) followed by dehydration overnight in absolute ethanol. Using a small brush, samples are washed in a small Petri dish, stained for 2 h with Feulgen's reagent, washed again, dehydrated, and cleared with xylol. Infected acini that appear as Feulgen-positive bodies (DNA red-purple and cytoplasm green) can be quantified by immersion in xylol or methyl salicylate in a Petri dish or after slide mounting in Canada balm or DPX using a stereomicroscope at magnifications of ×500 or higher. Other, more time-consuming staining methods such as green-methyl pyronine, toluidine blue, or hematoxylin-eosin (histological sections) can also be used.

Molecular Detection

Samples and storage

Both blood and spleen are the most commonly selected samples that are suitable for molecular detection of *Babesia* and *Theileria* spp. in vertebrate hosts. Samples should be stored at −20°C unless they are used within 72 h, in which case they should be refrigerated at +4°C. Spleen samples can also be stored in 70% ethanol. Under field conditions, blood samples can be dried onto FTA cards, which allows easy transportation and long-term storage (Rahikainen et al. 2016).

Prevalence studies of *Babesia* and *Theileria* in ticks should be based on analysis of questing ticks collected from the environment, as positivity in ticks collected from hosts may simply represent a remnant of the most recent bloodmeal rather than an active infection in the tick. All stages of ticks can be used for epidemiological studies, but it should be

Table 1. Morphological Description and Geographic Distribution of *Babesia* and *Theileria* Species Occurring in Different Vertebrate Hosts

Babesia/Theileria *species*	*Size*	*Typical parasi-taemia (%) in clinical cases*	*Characteristic features*	*Geographic distribution*	*Reference*
Species reported from humans					
Babesia divergens	"Small" 1.9×0.8 μm (piriforms)	Up to 80%	Ring stages characterized by big vacuoles; merozoites subcentral; tetrad stages; and polyparasitism (5–8 parasites per RBC) common	Throughout Europe and North Africa	Zintl et al. (2003); Figueroa et al. (2010)
Babesia venatorum (EU1)	"Small" 1–2.5 μm (ring stages)	1–30%	Ring forms most common; tetrad stages present but rare; position in RBC's generally peripheral	Cases reported from Italy, Austria, Germany, and China	Häselbarth et al. (2007); Herwaldt et al. (2003); Jiang et al. (2015)
Babesia microti	"Small" 1–2.5 μm	4.5%	"Characteristic" ring and piriforms; parasites usually present singly or in pairs	Germany (single case); USA	Hildebrandt et al. (2007); Smith et al. (2014)
Babesia Duncani	"Small"	6%	Round to oval, with some piriform, ring, and ameboid forms	USA	Conrad et al. (2006)
Bovine SPP					
Babesia bigemina	"Large" 2.5–3.5 μm (ring stages) 2.5–4.5 μm (piriforms) (may extend to the full diameter of the RBC)	Up to 40%	Single forms, elongated or amoeboid with fine cytoplasmic filaments; paired piriforms typically at an acute angle	Tropical and subtropical regions of all continents, including Southern Europe	De Vos and Potgieter (1994); Bock et al. (2004); Mosqueda et al. (2012); Figueroa et al. (2010)
Babesia bovis	"Small" 1–1.5 μm (ring stages) 1.5–2.4 μm (piriforms)	1–0.1% (highest in capillary blood)	Single forms, generally round or oval; paired piriforms, usually but not always at an obtuse angle and less common than vacuolated signet ring forms; amoeboid forms rare	As for *B. bigemina* but less widespread	De Vos and Potgieter (1994); Bock et al. (2004); Mosqueda et al. (2012); Figueroa et al. (2010)
B. divergens	"Small" 1.5–1.8 μm (ring stages) 1.5–1.9×0.4–1.1 μm (piriforms)	Up to 30–45%	Widely divergent paired organisms at the very edge of RBC's, may cause slight protrusions of the erythrocyte membrane; tetrad formations and polyparasitism infrequent	Europe and North Africa	Zintl et al. (2003)
Babesia major	"Large" 1.8 μm (ring stages) 2.6×1.5 μm (piriforms)	No information available	Piriforms characteristically paired at an acute angle; position typically central	Europe	Bock et al. (2004); Figueroa et al. (2010)
Theileria parva	No information available	No information available	Schizonts ("Koch bodies") typically with 12 nuclei (up to 80 reported from in vitro cultures) Piroplasms predominantly round or oval; division by binary fission may result in two or four daughter cells, the latter typically in the shape of a cross	East Africa	Morrison (2015)

(*continued*)

TABLE 1. (CONTINUED)

Babesia/Theileria *species*	*Size*	*Typical parasi-taemia (%) in clinical cases*	*Characteristic features*	*Geographic distribution*	*Reference*
Theileria annulata	1–15 μm (average 8, max. 27 μm) (schizonts in macrophages/monocytes)	May reach 95%	Schizonts (''Koch bodies'') typically with 12 nuclei (up to 80 reported from in vitro cultures)	North Africa, Mauritania, Sudan, Near and Middle East, central Asia, Indian subcontinent, southern	Mehlhorn (2008); Uilenberg (1981); Darghouth et al. (2010); Pipano and Shkap (2004)
	In RBC's: 0.5–2 μm (round stages); 0.5 × 1.6 μm (comma shapes); parasites observed during the acute stage smaller than those seen during the chronic stage of infection		Piroplasms predominantly round or oval; division by binary fission may result in two or four daughter cells, the latter typically in the shape of a cross	Europe (Portugal, Spain, the Balkans)	
Theileria buffeli/ orientalis complex	Highly variable	No information available	Variable morphological features; predominantly bacilliform; piroplasms sometimes associated with a ''bar'' or ''endoerythrocytic veils''; spiny deformation of RBC's during acute infection	Asia, Mediterranean basin, western Europe, Australia, North Africa, North America?	Mehlhorn (2008); Darghouth et al. (2010); Lawrence (2004a)
Canine SPP					
Babesia canis	''Large'' 2.5–3 × 5 μm (piriforms) 2–4 μm (amoeboid forms)	1%	Piriform, usually paired; polyparasitized RBC's common (up to 16 parasites/RBC)	Europe, Asia	Solano-Gallego and Baneth (2011); Ayoob et al. (2010a); Purnell (1981)
Babesia vogeli	''Large'' 2.5 × 4.5 μm	No information available	Piriform, single or paired	Tropical and subtropical regions of all continents, including southern Europe	Solano-Gallego and Baneth (2011); Ayoob et al. (2010a); Figueroa et al. (2010)
Babesia rossi	''Large'' 2.5 × 4.5 μm	No information available	Piriform, single or paired	Africa; reported mostly from southern Africa and also from Nigeria and Sudan	Jacobson (2006); Solano-Gallego and Baneth (2011)
Babesia gibsoni	''Small'' 1.2 × 3.2 μm (ring stages) 1.4–2 μm (piriforms) 2–2.5 μm (amoeboid forms) (no more than one eighth the diameter of the host erythrocyte)	No information available	Pleomorphic with oval and signet rings, single or in pairs; tetrads observed occasionally	Southeast Asia, USA, South America, Europe	Solano-Gallego and Baneth (2011); Ayoob et al. (2010a); Purnell (1981)
Babesia vulpes	''Small'' 2.5 × 1 μm	No information available	Typically single	Northern Spain, Portugal, Croatia, Sweden	Baneth et al. (2015); Ayoob et al. (2010a); Zahler et al. (2000)

(*continued*)

Table 1. (Continued)

Babesia/Theileria *species*	*Size*	*Typical parasi-taemia (%) in clinical cases*	*Characteristic features*	*Geographic distribution*	*Reference*
Babesia conradae	"Small"	No information available	Morphologically not distinguishable from other canine small *Babesia* spp. by light microscopy	California	Kjemtrup et al. (2006)
Cervine SPP					
Babesia capreoli	"Small" (1–3 μm), larger in fallow than in roe deer RBC's	20%–25% in roe and reindeer	Round to oval of various sizes; paired piriforms divergent and peripheral; tetrads and polyparasitism (≥8 parasites per RBC) present in roe, not in fallow deer	Throughout Europe	Malandrin et al. (2010)
B. divergens		Isolated cases in reindeer		Throughout Europe and North Africa	Wiegmann et al. (2015)
B. venatorum	No information available	Deer	No information available	Throughout Europe	Herwaldt et al. (2003)
Equine SPP					
Babesia caballi	"Large" 1.5–3 μm (ring stages) 2–5 × 1–1.5 μm (piriforms)	≤0.1%	Piriforms typically in pairs with an acute angle between them; RBC's rarely with >2 parasites	Africa, South and central America and southern USA, southern Europe, Asia	Bock et al. (2004); Wise et al. (2013); De Waal and Heerden (2004)
Theileria equi	"Small" 2–3 μm in diameter	1–5% (severe cases may exceed 20%)	Polymorphic (oval, round, elliptical, or piriform); typically two or four piriforms per RBC; distinctive Maltese cross formation	Southern Europe Africa, Asia, America	Wise et al. (2013); Mehlhorn (2008); De Waal and Heerden (2004)
Feline SPP					
B. canis	"Large" 3 × 5 μm	No information available	Piriform, singlet and pairs	Spain, Portugal	Ayoob et al. (2010b)
Babesia felis	"Small" 0.9 × 0.7 μm	Determined by immunocompetence, chronicity of infection, and concurrent disease	Single or paired annular body (signet rings), pair-shaped forms, and rarely tetrads	Africa, South Asia, Europe	Ayoob et al. (2010b)
Babesia lengau	"Small" 1.1 × 1.9 μm	No information available	Found in the blood and also in brain capillaries associated with feline cerebral babesiosis	Africa	Bosman et al. (2013)
Babesia presentii	"Large" 2.5 × 1.4 μm	1–5%	Single ring forms and paired merozoite forms, with some cells containing four merozoites not arranged as tetrads	Israel	Baneth et al. (2004)

(*continued*)

Table 1. (Continued)

Babesia/Theileria *species*	*Size*	*Typical parasi-taemia (%) in clinical cases*	*Characteristic features*	*Geographic distribution*	*Reference*
Ovine and caprine SPP					
Babesia motasi	"Large" 2.2–3.8 × 2 μm (piriforms) (occupying two thirds or more of the RBC)	No information available	Usually piriform, singly or in pairs, generally at an acute angle; ring, oval, elongated, or budding forms less frequent; forms with two nuclei common	Europe, particularly the Mediterranean basin, Africa, Asia, America? (Cuba?)	Bock et al. (2004); Figueroa et al. (2010); Yeruham and Hadani (2004)
B. ovis	"Small" 1–2.5 μm	5–7.5%	Mostly oval or piriform, paired forms usually at an obtuse angle; position in RBC's typically marginal; tetrads uncommon; all forms with single nucleus	Mediterranean basin, Balkans, Kazakhstan, Kirghizstan, Turkmenistan, Iraq, Iran	Bock et al. (2004); Yeruham and Hadani (2004)
Theileria lestoquardi (syn. *T. hirci)*	10–20 μm (average 8 μm) (schizonts in macrophages/ monocytes) In RBC's: 0.6–2 μm (round stages); 1.6 μm (comma shapes)	Parasitaemia typically much higher than in *Theileria ovis*	Round, oval, or rod shaped	Balkans, Mediterranean basin, Middle East, North and East Africa, Asia	Mehlhorn (2008); Darghouth et al. (2010); Lawrence (2004b)
T. ovis (syn. *Theileria recondita, Theileria sergenti*)	As for *T. lestoquardi*	<2% (of RBC's)		Widespread in Africa, Asia, and Europe	Mehlhorn (2008); Darghouth et al. (2010); Lawrence (2004b)
Porcine SPP					
Babesia perroncitoi	"Small" 0.7–2 μm (ring stages) 1.2–2.6 × 0.7–1.9 μm (piriforms)	Up to 60%	Mostly pleomorphic and annular (some oval, quadrangular, and piriforms also occur), usually single, sometimes ≥2 per cell	Southern Europe (Italy), West and Central Africa, China, Vietnam	Bock et al. (2004); Figueroa et al. (2010); De Waal (2004)
Babesia trautmanni	"Large" 2.5–4 × 1.5–2 μm (piriforms)	Up to 60%	Paired forms, usually oval or pirifoms; single forms, typically amoeboid or round; 1–4 parasites per RBC	Southern Europe, former USSR, Africa	Bock et al. (2004); Figueroa et al. (2010); De Waal (2004)

RBC, red blood cell.

remembered that *Theilera* species do not undergo a transovarial transmission in the vector tick whereas some *Babesia* species do, and therefore larvae are not recommended for detection of *Theileria* species (Schnittger et al. 2012). Whenever epidemiological studies in ticks are performed, it is of utmost importance to identify the collected ticks to species level to gain valid epidemiological data and to avoid drawing wrong conclusions regarding host–pathogen associations.

Ticks can be stored in 70% ethanol at room temperature or +4°C, or they can be frozen at −20°C or −80°C depending on the intended usage. Analyses may be performed on partial or whole ticks, individually or pooled, depending on the study design.

DNA, RNA, and protein extraction

Before DNA extraction, whole ticks may be split into two longitudinal halves using a sterile scalpel blade. One half can then be processed further, whereas the other could be stored as a back-up sample. Longitudinal slicing is especially recommended for fully engorged ticks, as the relatively large amount of sample can interfere with the DNA extraction process. Individual organs may also be dissected and analyzed to address more specific research questions: Analysis of SG is recommended for assessing pathogen transmission capacity; analysis of ovaries for investigating vertical transmission; and midgut analysis to track pathogens after meals of infected blood. SG, ovaries, and midgut are isolated using the method described earlier. The gut is a dark red, spider-shaped structure. On removal of the gut, ovaries appear as an inverted U-shaped structure distal to the rectal sac (Edwards et al. 2009).

Before extraction, all tick tissue samples, whether whole or dissected parts, as well as host spleen samples, should be homogenized either manually with a sterile scalpel blade or automatically with a device such as the Tissue Lyser (Qiagen) or Precyllis® (Halos et al. 2004). Afterward, samples are directly placed in DNA extraction buffer (according to the chosen commercial kit) or in RNA later® or Trizol® if RNAs and proteins are also to be extracted.

Total DNA extraction of all tissues mentioned earlier and blood can be performed using a commercial DNA extraction kit, although other methods using Proteinase K have been described (Boom et al. 1990). Extracted DNA can be stored at +4°C (short term) or −20°C (long term) until further use. The quality and quantity of extracted DNA can be evaluated using gel electrophoresis, spectrophotometry, or partial gene amplification of conserved genes of the tick or vertebrate host (18S, β-actin, ITS, or others).

PCR analysis

Several conventional and real-time PCR assays have been described for detection of *Babesia* and *Theileria* spp. in vertebrate and tick hosts. These techniques are usually more sensitive than microscopy (Wang et al. 2015) and, depending on gene target and size, they may permit identification to genus or species level and/or phylogenetic analysis. Genus-specific assays are recommended for epidemiological studies when several piroplasm species may be present, or for diagnostic purposes when species-specific assays fail, but piroplasms are suspected. Finally, genus-specific assays are used when a piroplasm that cannot be further identified is found. In the latter case, full sequence analysis of the 18S rRNA should be attempted. On the other hand, species-specific molecular methods are used for diagnostic purposes or for epidemiological studies of the distribution of a particular piroplasm species.

Table 2 lists primers used for genus and species-specific detection and identification of *Babesia* and *Theileria* spp. Molecular targets include the 18S rRNA gene, HSP70, ITS1, CCTeta, Ema-1, and Tams, with the 18S gene being, by far, the most commonly used target gene. Nuclear ribosomal rRNA genes are frequently used as targets for species identification (Chae et al. 1998, Katzer et al. 1998, Allsopp and Allsopp 2006), because of their conserved nature and repetitive arrangement within the genome that provide ample amounts of template DNA for PCR. Although a high degree of 18S rRNA gene sequence conservation has been reported between *Babesia* and *Theileria* species, it has been recommended that the complete 18S rRNA gene be amplified, particularly when dealing with new organisms, to ensure that genetic variation is not overlooked (Herwaldt et al. 2003, Hunfeld et al. 2008, Bhoora et al. 2009).

Tams 1 encodes a major polypeptide that is located on the surface membrane of merozoite and piroplasm stages of *Theileria annulata*. Because of the level of diversity within the gene, the primers listed may not detect all *T. annulata* genotypes, potentially leading to an underestimation of the prevalence of this pathogen (Katzer et al. 2006, Santos et al. 2013).

Alternative molecular methods include reverse line blot, which allows simultaneous screening of large numbers of pathogens using probe hybridization (Gubbels et al. 1999, Hurtado 2015), and modern high-throughput screening methods such as the microfluidic real-time PCR system (Fluidigm) (Michelet et al. 2014) and Next Generation sequencing (Bonnet et al. 2014).

In Vitro Culture

With the widespread use of molecular tools, traditional methods such as in vitro culture are used less frequently in current research. It could be argued, however, that without detailed information on parasite biology and its interaction with the host, molecular data are of limited use. Cell culture-based research is an important tool for gaining such information, for allowing for easy manipulation of conditions, and for reducing the need for animal experimentation.

The intra-erythrocytic stage of a great number of *Babesia* species has been established in in vitro culture (Table 3). In contrast, most successful in vitro culture systems for *Theileria* spp. involve the pre-erythrocytic schizont stage in leukocytes (Table 4).

In vitro culture of Babesia *and erythrocytic* Theileria *stages*

The earliest in vitro methods used to grow *Babesia* parasites relied on suspension cultures adapted from systems originally developed for *Plasmodium* (Trager and Jensen 1976). However, these required large volumes of reagents and extensive manipulations and were quickly replaced by microaerophilous stationary-phase systems, which are characterized by reduced O_2 tension in the atmosphere, and a static layer of erythrocytes settled at the bottom of the culture unit (Levy et al. 1981). Since then, a variety of methods have

Table 2. Primers Designed for Polymerase Chain Reaction and Real Time PCR Detection of *Babesia/Theileria* Species

Organism	*Target gene*	*Amplicon size (bp)*	*Primers*	*Reference*
Apicomplexa	18S rRNA	1700	CryptoF: AACCTGGTTGATCCTGCCAGT CryptoR: GCTTGATCCTTCTGCAGGTTCACCTAC	Herwaldt et al. (2003)
Babesia/Theileria spp.	18S rRNA	411–452	BJ1: GTCTTGTAATTGGAATGATGG BN2: TAGTTTATGGTTAGGACTACG	Casati et al. (2006); Lempereur et al. (2011)
Babesia/Theileria spp.	18S rRNA	389–426	BTV4_Fow: CACAGGGAGGTAGTGACAAG BTV4_Rev: AAGAATTTCACCTCTGACAG	Schnittger et al. (2004)
Babesia spp.	18S rRNA	422–440	BabsppF1: GTTTCTGMCCCATCAGCTTGAC BabsppR: CAAGACAAAAGTCTGCTTGAAAC	Hilpertshauser et al. (2006)
B. venatorum	18S rRNA	91	Bab_EU_RNA18S_F: GCGCGCTACACT GATGCATT Bab_EU_RNA18S_R: CAAAAATCAATC CCCGTCACG Bab_EU_RNA18S_P: CATCGAGTTTAAT CCTGTCCCGAAAGG	Michelet et al. (2014)
B. divergens	Hsp 70	83	Bab_di_hsp70_F: CTCATTGGTGACGCCGCTA Bab_di_hsp70_R: CTCCTCCCGATAAGCCTCTT Bab_di_hsp70_P: AGAACCAGGAGGCCCGT AACCCAGA	Michelet et al. (2014)
Babesia microti/ Babesia duncani	ITS1	930–950	BABITS_F: GGTGAACCTGCRGAAGGATC BABITS_R: TCTKCCGCTTARTTATATGC	Wilson et al. (2015)
B. microti	CCTeta	145	Bab_mi_CCTeta_F: ACAATGGATTTTCC CCAGCAAAA Bab_mi_CCTeta_F: GCGACATTTCGGCAA CTTATATA Bab_mi_CCTeta_P: TACTCTGGTGCAATGA GCGTATGGGTA	Michelet et al. (2014)
Babesia ovis	18S rRNA	549	OvisB_Fow: TGG GCA GGA CCT TGG TTC TTC OvisB_Rev: CCG CGT AGC GCC GGC TAA ATA	Aktas et al. (2005)
T. equi	Ema-1	115–270	Ema 1-f: GAGTCCATTGACCACCTCAC Ema 1-r: GTGCCTGACGACAGTCTTTGG Ema 1-p: TCGACAAGCAGTCCGAGCACA	Ueti et al. (2003)
Babesia caballi	Ema-1	700	BC 48-f: ACGTACACGAATGTTGATGAGTT BC 48-r: AGAGCGAATAATCTGCTTAAGTGC BC 48-p: TGATGCCCCAGCCGAACAACCCAG	Heim et al. (2007)
Theileria spp.	18S rRNA	230	F: GGTAAT TCCAGC TCC AATAG R: ACC AAC AAA ATAGAACCA AAG TC	Sibeko et al. (2008)
T. annulata	*Tams1*	319	Tams1F: CCAATTCGAGACCTACTACGATG Tams1R: CCACTTRTCGTCCTTAAGCTCG	Santos et al. (2013)

been developed, some of which are detailed in Table 3. As the table indicates, there are some differences of opinion between laboratories on the optimum culture conditions for any given species. This may be due to regional differences in parasite strains and their specific requirements or variations in the suitability of sera and red blood cells derived from donor animals (Canning and Winger 1987). The methods described here are based on culture systems originally developed by Holman et al. (1993a). In our experience, it supports the isolation and maintenance of many *Babesia* species, including some of the most fastidious members of the genus, as well as *Theileria equi* and *T. uilenbergi*, the only two *Theileria* spp. that have been successfully maintained in the erythrocytic stage (Holman et al. 1994a, Zweygarth et al. 1995, Miranda et al. 2006).

Given appropriate culture conditions, the parasites usually reach optimal growth within one or two subcultures after isolation, suggesting that a period of adaptation and/or selection is not required (Levy et al. 1981, Canning and Winger 1987).

(1) Donor red blood cells:

Anticoagulants have an inhibitory effect, and best culture growth is usually achieved with defibrinated erythrocytes. However, red blood cells collected into anticoagulant can be used if they are subjected to thorough washing before use.

(a) Preparation of defibrinated erythrocytes:

Defibrinated red blood cells are prepared by collecting blood from a donor animal directly into an air-tight sterile conical flask containing glass beads (0.5 to 1 cm in diameter) and swirling gently and continuously until a clot has formed on top of the liquid. After centrifugation (500 *g*, 10 min, 4°C), and removal of the serum and buffy layer (upper third of the cell pellet), the lower half of the remaining pellet is carefully withdrawn, transferred to an equal volume of Puck's saline (Puck's saline [g/L]: NaCl: 8; KCl: 0.4; $MgSO_4 \cdot 7H_2O$: 0.154; $CaCl_2 \cdot 2H_2O$: 0.016; $Na_2HPO_4 \cdot 7H_2O$: 0.29; KH_2PO_4: 0.15; glucose: 1.10; phenol red: 0.0012.) (with 2% extra glucose), and stored at 4°C. The shelf-life of red blood cells in Puck's saline ranges from about 2 to 5 weeks depending on

Table 3. Culture Conditions Developed for Various *Babesia* spp. and Erythrocytic *Theileria* Merozoites

Species	*RBC compatibility*	*Serum compatibility*	*Suitable culture media*	*Reference*
B. bigemina	Bovine (5–10% PCV)	20–50% adult bovine serum	Medium 199 in Hank's (with 2.2 g/L $NaHCO_3$) or in Earle's balanced salt solution (with 1.4 g/L $NaHCO_3$) or HL-1 with 2 mM L-glutamine	Canning and Winger (1987); Vega et al. (1985); P. Holman (personal communication)
B. bovis	Bovine (PCV most commonly 5%, 9% in older publications) Can be adapted to grow in horse, rabbit, and deer RBC's	20–40% adult bovine serum Horse and rabbit serum (cervine serum is inhibitory)	Medium 199 in Hank's balanced salt solution with 15 mM HEPES (pH 7); Medium 199 in Earle's balanced salt solution with 20–26 mM TES (pH 7), ±2 mM L-glutamine; or RPMI 1640 with 15 mM HEPES (pH 7) or HL-1 with 2 mM L-glutamine	Canning and Winger (1987); Timms et al. (1983); Palmer et al. (1982); Ristic and Levy (1981); Holman et al. (1993b); P. Holman (personal communication)
B. caballi	Equine (5% or 9% PCV)	20–40% adult horse serum	HL-1 with 2 mM L-glutamine; or RPMI 1640 with 20 mM HEPES	Holman et al. (1993a); Avarzed et al. (1997)
B. canis	Canine (2% PCV)	10–20% adult dog serum	RPMI 1640 with 20–40 mM HEPES (±0.6 g/L reduced glutathione) (pH 7.2–7.4)	Schetters et al. (1997); Canning and Winger (1987)
B. capreoli	Exclusively in roe deer (7.5% PCV)	20% FCS	RPMI 1640	Malandrin et al. (2010)
B. divergens	Good growth in bovine, human RBC's, moderate growth in sheep (5–7.5% PCV), slow growth in roe deer RBC's, very poor growth in horse RBC's	10–30% bovine (calf and adult) or sheep serum (horse serum was always inhibitory, and human serum was sometimes found to be inhibitory)	HL-1 medium with 2 mM L-glutamine; or RPMI 1640	Zintl et al. (2002); Malandrin et al. (2004); Grande et al. (1997)
B. gibsoni	Canine (10% PCV)	20–40% adult dog serum	HL-1 medium with 15 mM HEPES, 2.2 g/L $NaHCO_3$, 2 mM L-glutamine, 0.2 mM hypoxanthine; or RPMI 1640 with 25 mM HEPES, 2 mM L-glutamine, 1 mM pyruvic acid, and 24 mM $NaHCO_4$	Zweygarth and Lopez-Rebollar (2000); Sunaga et al. (2002)
T. equi	Equine (10% PCV)	20% FCS or 40% horse serum	HL-1 with 1 g/L AlbuMAX 1, 1–2% HB101, 2 mM L-glutamine, ±16 μM thymidine (pH 7.3–7.4); or Medium 199 (with Hank's salts), 10 mM TAPSO, 2 mM L-glutamine, and 0.2 mM hypoxanthine (or 0.2 mM guanosine or adenosine)	Holman et al. (1994a, 1998); Zweygarth et al. (1995)
T. uilenbergi	Sheep (5–10% PCV)	20% sheep serum	HL-1 with 0.75% chemically defined lipid concentrate	Miranda et al. (2006)

FCS, fetal calf serum; PCV, packed cell volume.

Table 4. Culture Conditions for *Theileria* Schizonts

Species	*Host cells*	*Feeder layer*	*Serum compatibility*	*Suitable culture media*	*Reference*
T. annulata	Bovine (ovine and caprine) macrophages, dendritic cells, and B cells	Not required	20% FCS, normal bovine or goat serum	RPMI 1640; Leibovitz L-15	Brown (1987); Sharma et al. (1998); Tretina et al. (2015); Brown et al. (1998); Shkap et al. (1996)
T. lestoquardi (syn. *T. hirci*)	Ovine and caprine lymphocytes (mostly B cells)	Not specified	Ovine	Eagle's MEM based on HBSS, with lactalbumin hydrolysate and yeast extract	Hooshmand and Hawa (1975); Brown et al. (1998); Hooshmand et al. (1993)
T. parva	Bovine (and buffalo) B and T lymphocytes	BESP cells	10–20% (heat inactivated) FCS or newborn calf serum	RPMI 1640 with 20 mM HEPES and 2 mM L-glutamine or Eagle's MEM with 0.01 g/L L-asparagine or modified Leibovitz's L-15 medium (pH 6.8) with 10% tryptose phosphate	Jongejan et al. (1984); Malmquist et al. (1970); Stagg et al. (1974); Tretina et al. (2015); Kurtti et al. (1981)
Theileria taurotragi	Eland lymphocytes and apparently monocytes/ macrophages	BESP cells	20% FCS	Eagle's MEM	Stagg et al. (1976)
Theileria sp. (buffalo)	Buffalo lymphocytes	Bovine aortic endothelia cells	10% heat-inactivated FCS	RPMI 1640 with Glutamax; 25 mM HEPES, 2 g/L sodium bicarbonate; 0.2 mM hypoxanthine	Zweygarth et al. (2009a)
Theileria sp. (roan antelope)	Antelope lymphocytes	Not specified	10% heat-inactivated FCS	RPMI 1640, 25 mM HEPES, 2 g/L sodium bicarbonate; 0.2 mM hypoxanthine	Zweygarth et al. (2009b)

BESP, bovine embryonic spleen.

the donor species and is indicated by the degree of hemolysis in the supernatant.

(b) Preparation of erythrocytes collected into anticoagulant:

After centrifugation (500 *g*, 10 min, 4°C) and removal of the plasma and buffy layer, the cell pellet is washed twice in Dulbecco's phosphate-buffered saline (DPBS) (pH 7.2) with 15 mM EDTA, and once in Puck's saline (with 2% extra glucose) with complete removal of the buffy layer after each wash. Subsequently, the bottom third of the pellet is carefully withdrawn and added to an equal volume of Puck's saline (with 2% extra glucose). Shelf-life and recommended storage conditions are as described earlier.

(2) Culture media:

As first described by Holman (1993a) and later confirmed in several other in vitro systems (Holman et al. 1994a, 1994b, 1994c, Zweygarth and Lopez-Rebollar 2000, Zintl et al. 2002), HL-1 medium (BioWhittaker UK) supports the growth of *Babesia and Theileria* spp. extremely well. This medium, which was originally designed as a serum-free, chemically defined medium for in vitro cultivation of hybridoma and other cells of lymphoid origin, is usually supplemented with serum from an appropriate naïve host animal. Heat inactivation of the serum is not necessary. In fact, early workers suggested that complements assisted erythrocyte invasion by the parasite (Chapman and Ward 1977). For many *Babesia* spp., excellent culture growth has been achieved with fetal calf serum (FCS)-supplemented medium. Notable exceptions are *Babesia bigemina*, *B. bovis*, *Babesia divergens*, and *Babesia caballi*, which grow better in the presence of normal serum.

(3) Culture initiation:

In our experience, culture isolations have a better chance of success if they are initiated using infected red blood cells rather than merozoites separated from the host cell, as the latter have reduced viability and tend to clump together. In most cases, infected red blood cells are collected in small volumes from an infected animal, and because the amount is small, the blood is mixed with anticoagulant rather than defibrinated. After centrifugation (500 *g*, 10 min, 4°C), plasma and the buffy layer are removed. The cells are then washed twice in five volumes of cold DPBS with 15 mM EDTA and once in DPBS without EDTA (with removal of the buffy layer after each centrifugation). Although thorough washing is essential to remove all traces of host plasma (including potential immune mediators) and, in the final step, anticoagulant, it is important to proceed gently, particularly if dealing with species that increase red blood cell fragility, such as *B. divergens* and *B. bigemina* (Canning and Winger 1987). Subsequently, 0.05 to 0.1 mL of washed infected erythrocytes are added to 1 to 1.1 mL HL-1 medium (supplemented with 2 mM L-glutamine and serum) in each well of a lidded 24-well plate. The exact amount of infected red blood cells that is required to initiate the culture depends on the parasitaemia in the sample. It is also dependent on whether the infected red blood cells are derived from the same or a different species, as mixing of heterologous material can lead to hemolysis, thereby leading to a decrease in the proportion of red blood cells or packed cell volume (PCV). If necessary, the red blood cell volume in the culture wells is adjusted to the desired PCV (Table 1) by the addition of donor erythrocytes. Standard concentrations of antibiotics may also be added to control the growth of bacterial and fungal contaminants.

(4) Culture maintenance:

Cultures are maintained in 24-well plates in a total volume of 1.1 to 1.2 mL culture mix per well consisting of HL-1 medium (supplemented with 2 mM L-glutamine and serum) and donor erythrocytes at a PCV of between 1% and 10%. It is important to note that different species have specific preferences for PCV (Table 3), as erythrocyte concentrations lower or higher than the optimum can suppress culture growth. Depending on requirements, it is, of course, possible to either scale up or down culture systems by changing the well size; however, care must be taken to maintain the same PCV and surface-to-volume ratio. Good culture growth requires a low O_2 concentration in the erythrocyte layer (Rodriguez et al. 1983). In new or slow-growing cultures, this is usually achieved by incubating the cells in a microaerophilous atmosphere consisting of 5% CO_2, 2% O_2, and 93% N_2 (provided by commercial suppliers) in airtight modular incubation units (or hypoxia chamber, Billups-Rothenberg). The chambers are equipped with an inflow that can be connected to the gas cylinder and an outflow. After flushing of the chamber with the special gas mix, both openings are sealed and the chamber is placed in the incubator. Humidity is provided by pipetting a small amount of sterile water into the bottom of the chamber. In well-established, fast growing cultures, the metabolism of parasites is sufficient to deplete the O_2 concentration in the erythrocyte layer. As a result, these cultures can be maintained at 5% CO_2 in air [in fact, in some established culture systems, transfer to CO_2 in air has been reported to boost in vitro growth (Avarzed et al. 1997)]. It is important to ensure, however, that the depth of the medium is maintained at a level that does not allow for complete replenishment of O_2 in the settled layer (around ≥ 6.2 mm but optimization may be required). The supernatant is replaced daily with fresh medium, and parasite growth is monitored by microscopic examination of fixed and Giemsa-stained smears prepared from the erythrocyte layer. Optimal subculturing and maintenance conditions are determined by allowing a culture to ''grow out'' and recording percentage of infected red blood cells daily. Subcultures should be carried out during optimum exponential growth by transferring resuspended cultures to freshly prepared wells to achieve parasitaemias of $\leq 1\%$. Optimum incubation temperatures vary slightly depending on the normal body temperature of the respective host species.

(5) Storage:

(a) Short-term storage: Once continuous cultures are established, isolates can be switched to ''slow growth'' between experiments by transferring infected blood cells in culture medium to 4°C (Canning and Winger 1987). Although parasitaemia falls off rapidly within the first few days at 4°C, this decline is slowed down by weekly changes of medium. Normal growth resumes once cultures are returned to the incubator.

(b) Cryopreservation: For long-term storage, cultures can be transferred to liquid nitrogen (N_2). Stabilates are prepared from healthy cultures during exponential growth. After centrifugation (500 *g*, 10 min, 4°C) and removal of the supernatant, the pellet is resuspended in an equal volume of ice-cold Puck's saline supplemented with 2% glucose and 20% polyvinylpyrrolidone-40. The suspension is dispensed into cryovials and immediately transferred to −70°C. After 20 to 30 h at −70°C, the cultures are placed into liquid N_2. The cultures are revived by rapid thawing and transferred to freshly prepared culture wells containing complete medium and donor erythrocytes.

In vitro *culture of* Theileria *schizonts in lymphoblastoid or monocytic cells*

This technique exploits the ability of certain *Theileria* species to induce a cancer-like immortalized leukocyte phenotype that can proliferate indefinitely (Brown 1987). During each mitosis, schizonts that reside in the host cell cytosol bind to the host mitotic spindle, ensuring segregation of the parasite into both daughter cells (Tretina et al. 2015). All *Theileria* spp. that have been shown to have the ability to elicit this transformation (*Theileria parva*, *T. annulata*, *Theileria lestoquardi*, *Theileria taurotragi*, *Theileria* sp. [buffalo]) (Sivakumar et al. 2014) have been established in immortalized schizont-infected cell lines (Brown 1987); whereas species such as *T. mutans*, *T. sergenti*, *T. velifera*, and *T. cervi* seem to have, to our knowledge, little or no transforming ability and have never been established in schizont cultures.

(1) Culture initiation

Cultures can be initiated using lymphoid tissue or peripheral blood mononuclear cells (PBMCs) from infected animals or sporozoites isolated from engorged ticks.

(a) Suitable lymphoid tissues include lymph node, spleen, thymus, bone marrow, lung, liver, and kidney (Brown 1987). After washing in complete medium containing antibiotics and preservative-free heparin (10 IU/mL), the tissue is homogenized to produce a cell suspension that is adjusted to a concentration of 2×10^6 cells/mL and plated (10 mL/25 cm^2 flask; 2 mL/2 cm^2 well; or 1 mL/1 cm^2 well). Alternatively, infected PBMCs are isolated from whole blood or the buffy layer either by erythrocyte lysis (in ice-cold 0.17 M ammonium chloride) or by density gradient centrifugation (using Ficoll-Paque, Histopaque, or Lymphoprep, each of which is formulated to a density of 1.077) (Brown 1987, Zweygarth et al. 2009a). Subsequently, isolated PBMCs are washed in EDTA/PBS (pH 7.2) or autologous plasma, resuspended in complete medium, and plated. During acute infections, cultures may be initiated using whole blood instead of PBMCs (Gharbi et al. 2012). Confusion of the cellular origin of the cell line or overgrowth of certain cell populations in the heterogenous cell mixture derived from tissue or blood can be avoided by enriching the cell suspension for monocytes or lymphocytes (depending on the host cell preference of the *Theileria* spp.). This is done by allowing the monocytes in the suspension to attach to the culture flask (''preculture'' for 3 h at 37°C) and discarding or transferring the unattached cells to a new flask (Kurtti et al. 1981). Uninfected lymphocytes have negligible mitotic activity and are eventually diluted out of the cultures when the lymphoblastoid cells are passaged.

(b) Cultures could also be initiated by introducing sporozoites harvested from infected ticks into uninfected mononuclear cells from susceptible animals (Kurtti et al. 1981, Brown 1987, Kimbita et al. 2004). After allowing infected adult female ticks to engorge to stimulate sporozoite maturation, they are surface sterilized using 1% benzalkonium chloride (one wash) and 70% ethanol (three washes). Subsequently, whole ticks or aseptically collected SG (if infection levels are low) are ground in ice-cold culture medium; the supernatant is centrifuged, filtered to remove debris, and used to infect mononuclear cells that are isolated as described earlier.

For most species, pre-established monolayers of supporting feeder cell lines of bovine embryo spleen, bovine embryo thymus, bovine embryo skin, bovine aortic endothelium, or buffalo lung cells (IMR31), together with their conditioned medium, provide unspecified growth factors, greatly enhancing the chances of successful culture initiation (Brown 1987).

(2) Culture maintenance

Although recent studies continue to use traditional cell culture media such as Eagle's MEM, RPMI 1640, and Leibovitz's L-15 medium, it is likely that some of the modern media available today such as HL-1 or Opti-MEM may negate the need for a feeder layer. Generally, media are supplemented with antimicrobials, antimycotics, and FCS, although Sharma et al. (1998) managed to maintain *T. annulata* long-term cultures in media containing normal bovine or goat serum. Cultures are maintained at 37°C in 5% CO_2 in air, and media are changed every other day.

Depending on the host cell, cultures are grown as monolayers or in suspension and monitored using Giemsa-stained cytospin smears (Sharma et al. 1998, Gharbi et al. 2012). Growth parameters include the ''schizont index'' (infected WBC's/examined WBC's), the ''mitotic index'' (mitotic cells/infected cells), and mean schizont nuclear number (number of theilerial particles per cell) (Sharma et al. 1998, Gharbi et al. 2012). As a rule of thumb, schizonts of both *T. annulata* and *T. parva* double every 18–21 h, necessitating subculture every 3 days (after a 10-fold multiplication).

(3) Cryopreservation

Theileria-infected cell lines can be frozen down for long-term storage essentially as described earlier for *Babesia* and *Theileria* merozoite cultures. Freezing media are composed of FCS and DMSO at a final concentration of 7% or glycerol at a final concentration of 10%. As DMSO is very toxic and immediately penetrates cell membranes, the freezing medium should be ice cold and the cryovials should be transferred to −70°C immediately. When glycerol is used, an equilibration time of 30–40 min is required before freezing the cultures. To initiate new cultures, frozen cells are rapidly thawed at 37°C and diluted 1:5 in complete medium before plating.

Xenodiagnosis Using Animal Inoculation

Inoculation of susceptible animals with whole blood from a suspected case is sometimes used to aid the diagnosis of human cases, although compared with molecular methods the procedure is time consuming, expensive, and questionable for ethical reasons. Most rodent laboratory hosts are susceptible to *B. divergens*, particularly if they have been splenectomized (Zintl et al. 2003); whereas infections are most reliably produced in intact Mongolian gerbils (Meriones unguiculatus) (Lewis and Williams 1979). For instance, the causative agent in a case of human babesiosis in Scotland was identified as *B. divergens* by inoculating a splenectomized calf with blood from the patient (Entrican et al. 1979), whereas inoculation of hamsters with whole blood from suspected cases has been used as a diagnostic method for *Babesia microti* (Kjemtrup and Conrad 2000). More recently, to aid the identification of newly discovered zoonotic agents such as *Babesia venatorum* and *Babesia Duncani*, the parasites were isolated in SCID mice and hamsters, respectively (Conrad et al. 2006, Jiang et al. 2015).

Conclusion

The past 50 years have seen the emergence of numerous methods for the detection and identification of *Babesia* and

Theileria parasites in the vertebrate and the tick hosts. Sample characteristics, research facilities, and infrastructure as well as worker experience and expertise will define the most practicable methods under any given set of circumstances. Nevertheless, we would like to urge researchers to consider using at least two independent methods wherever possible, as this would improve our understanding of the biological characteristics of many of the ''new'' species that have been identified, based chiefly on molecular data.

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