Parasitology Research (2020) 119:11–22
https://doi.org/10.1007/s00436-019-06557-7

PROTOZOOLOGY - REVIEW

An appraisal of oriental theileriosis and the *Theileria orientalis* complex, with an emphasis on diagnosis and genetic characterisation

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Received: 29 August 2019 / Accepted: 19 November 2019 / Published online: 6 December 2019

Abstract

Oriental theileriosis, a tick-borne disease of bovids caused by members of the *Theileria orientalis* complex, has a worldwide distribution. Globally, at least 11 distinct genotypes of *T. orientalis* complex, including *type* 1 (*chitose*), *type* 2 (*ikeda*), *type* 3 (*buffeli*), *types* 4 to 8, and N1–N3, have been described based on the sequence of the major piroplasm surface protein (*MPSP*) gene. Of these 11 genotypes, mainly *ikeda* and *chitose* are known to be pathogenic and cause considerable morbidity (including high fever, anaemia, jaundice and abortion), production losses and/or mortality in cattle. Mixed infections with two or more genotypes of *T. orientalis* is common, but do not always lead to a clinical disease, posing challenges in the diagnosis of asymptomatic or subclinical forms of oriental theileriosis. The diagnosis of oriental theileriosis is usually based on clinical signs, the detection of piroplasms of *T. orientalis* in blood smears, and/or the use of serological or molecular techniques. This paper reviews current methods used for the diagnosis of *T. orientalis* infections and the genetic characterisation of members of the *T. orientalis* complex, and proposes that advanced genomic tools should be established for investigations of these and related haemoparasites.

Keywords *Theileria orientalis* · Cattle · Buffalo · Ticks · Diagnosis · Characterisation

Introduction

Ticks and tick-borne diseases (TBDs) affect the productivity of bovines in tropical and subtropical regions of the world, leading to a significant socioeconomic impact on the livelihood of resource-poor farming communities (Makala et al. 2003). Globally, four main TBDs, namely anaplasmosis (primarily caused by *Anaplasma marginale* and *A. centrale*), babesiosis (*Babesia bovis*, *B. bigemina* and *B. divergens*), theileriosis (*Theileria annulata*, *T. parva*, *T. orientalis* complex and others) and heartwater/cowdriosis (*Ehrlichia ruminantium*) affect bovines, and cause substantial economic losses to cattle industry, particularly in the tropics and subtropics (Makala et al. 2003; Jabbar et al. 2015). The annual global costs of ticks and TBDs to cattle industry are estimated at US$14 to 19 billion worldwide (de Castro 1997). This impact is higher in developing countries where the costs of tick control and treatment strategies are almost unaffordable for low-income smallholder farmers (Minjauw and McLeod 2003; Rajput et al. 2006), particularly in many countries of sub-Saharan Africa, Asia and Latin America (Delgado et al. 1999).

Bovine theilerioses are caused by intracellular protistan parasites of the genus *Theileria* (Apicomplexa: Piroplasmida; Theileriidae) and are considered as one of the most economically important diseases of bovines globally (Uilenberg 1995). The geographical distribution of *Theileria* spp. is usually restricted to tropical and subtropical regions where suitable tick vectors occur. *Theileria* spp. infect primarily domestic and wild ruminants, and cause economically significant diseases in cattle, sheep and goats. For instance, *T. annulata* and *T. parva* (the causative agents of tropical or Mediterranean and East Coast

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Section Editor: Panagiotis Karanis

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fever, respectively) are known to be the most pathogenic species in bovines, whereas other species, such as *T. mutans*, *T. taurotragi* and members of the *T. orientalis* complex, often cause asymptomatic infections (cf. Jabbar et al. 2015). Depending on species of *Theileria*, a number of hard ticks of the genera *Amblyomma*, *Haemaphysalis*, *Hyalomma* and *Rhipicephalus* can transmit theilerioses.

Traditionally, theileriosis caused by *T. orientalis* complex was considered to be benign and asymptomatic, but clinical cases had been reported from Australia (Rogers and Callow 1966), Japan (Shimizu et al. 1992) and New Zealand (James et al. 1984). However, since 2010, a number of outbreaks of oriental theileriosis have occurred in Australia and New Zealand, leading to significant economic losses in dairy (Perera et al. 2014) and beef (Lane et al. 2015) cattle. Currently, 11 genotypes of *T. orientalis* are recognised, and only *ikeda* and *chitose* are known to be pathogenic, causing considerable morbidity and mortality in cattle. The diagnosis of oriental theileriosis can be made using approaches including clinical signs, the detection of piroplasms of *T. orientalis* in blood smears and/or the use of serological or molecular techniques. This article provides an account of oriental theileriosis and then reviews the current methods employed for the diagnosis of oriental theileriosis and the genetic characterisation of *T. orientalis*. It also highlights the need for advanced genomic tools for diagnostic and analytical applications.

The *Theileria orientalis* complex

The taxonomy and nomenclature of *T. orientalis* is still unresolved as different names are being used to describe similar parasites from different geographical locations, including *T. sergenti* from Japan, *T. buffeli* from Australia and *T. orientalis* from Europe and elsewhere (Watts et al. 2016). Recently, based on serological and morphological characteristics, Uilenberg (2011) proposed that *T. orientalis* represented a single species but suggested that it be called a complex. Different molecular markers, including the small subunit (SSU) of nuclear ribosomal RNA (18S rRNA), the first and second internal transcribed spacers (ITS-1 and ITS-2 = ITS) of nuclear ribosomal DNA, the cytochrome *c* oxidase III, 23-kDa piroplasm membrane protein (*p23*) and major piroplasm surface protein (*MPSP*) genes have been used to study and characterise members of the *T. orientalis* complex (Ota et al. 2009; Altangerel et al. 2011; Kamau et al. 2011a, b; Yokoyama et al. 2011, 2012; Perera et al. 2015a, b, c). Of these markers, the *MPSP* gene is the most commonly used marker; based on this gene's sequence, at least 11 distinct genotypes of *T. orientalis* (*types* 1–8 and N1-N3) from cattle, water buffaloes, sheep and ticks have been reported to date, worldwide (Sivakumar et al. 2014).

Distribution of *T. orientalis* complex

Oriental theileriosis caused by one or more genotypes of *T. orientalis* has become an important TBD of bovines, particularly in the Asia-Pacific region (Sivakumar et al. 2014). Recently, the first case of clinical oriental theileriosis was reported in cattle from Virginia, USA (Oakes et al. 2019). *Theileria orientalis* appears to be widely distributed globally, but in most countries, the genotypic identity of *T. orientalis* complex and the clinical relevance of the distinct genotypes is unclear. To date, *T. orientalis* has been recorded in bovines, sheep, ticks and other blood-feeding insects in more than 40 countries (Sivakumar et al. 2014). However, most reports originate from Japan, followed by Australia, China, Korea and New Zealand.

Life cycle of *Theileria orientalis*

The life cycles of *Theileria* spp. are indirect, involving ticks as vectors. Significant variation occurs in the life cycles of *Theileria* spp.; some species induce cell transformation and proliferation (e.g., *T. parva* and *T. annulata*), whereas others do not (e.g., *T. orientalis* complex). Generally, *Theileria* parasites have three distinct stages: (i) schizogony (asexual reproduction), which takes place in vertebrate hosts; (ii) gametogony (sexual reproduction)—the development and union of gametes inside the intestinal cells of tick vectors; and (iii) sporogony (asexual reproduction), which takes place in the salivary glands of tick vectors (Mehlhorn and Schein 1985; Nene et al. 2016). Sporozoites (the infective stage of the parasite) are inoculated with the saliva of a tick when it takes a blood meal from a vertebrate host. These sporozoites invade lymphoid cells and develop into a multinucleate schizont (e.g., in *T. annulata*) ~ 24 h after inoculation. Following merogony/schizogony, lymphocytes rupture, releasing merozoites which then enter erythrocytes. When a tick feeds on blood from the infected host, infected erythrocytes are ingested. Following ingestion, gametes are released into the gut lumen of the tick, where fertilisation occurs. Fertilisation gives rise to a spherical zygote that invades the gut epithelium of the tick. This zygote develops into motile ookinetes, which then invade the salivary gland of the tick, develop into sporoblasts and multiply asexually, producing thousands of sporozoites. Sporozoites are the infective stage and are injected when infected ticks feed on blood from another vertebrate host, thereby continuing the life cycle (Norval et al. 1992; Shaw 2003).

An ixodid tick, *Haemaphysalis longicornis* is the main vector of *T. orientalis* (see Watts et al. 2016). In addition, *H. bancrofti* and *H. humerosa* are also believed to be potential vectors in Australia (Stewart et al. 1987a, b; Watts et al. 2016). Additionally, other blood-sucking insects, such as March flies (tabanids), mosquitoes or sucking lice are also suggested as

potential vectors of *T. orientalis* (see Hammer et al. 2015, 2016; Watts et al. 2016). Furthermore, *T. orientalis* DNA has been detected in other tick species, including *Amblyomma variegatum*, *A. cohaerens*, *Rhipicephalus decoloratus*, *R. evertsi* and *R. praetextatus* in Ethiopia (Hornok et al. 2014; Kumsa et al. 2014), as well as from *R. annulatus* ticks in Italy (Toma et al. 2017). However, the competence of these vectors has not been established, as these ticks were collected from cattle, not from pastures, and no experimental infection with *T. orientalis* from these ticks has been demonstrated to date.

Pathogenesis and economic impact of oriental theileriosis

For many years, *T. orientalis* was recognised as a benign parasite of bovines (Watts et al. 2016), as its schizonts do not induce cell transformation or lymphoproliferation, unlike *T. annulata* and *T. parva*. Although the pathogenesis of *T. orientalis* is not yet completely understood (Watts et al. 2016), a rapid rate of division of both macroschizonts and microschizonts has been observed in calves infected with *T. orientalis* (Uilenberg et al. 1985), which highlights the potential of members of *T. orientalis* to be categorised as cell-transforming pathogens, but this hypothesis requires further testing.

Irrespective of the non-lymphoproliferative nature of *T. orientalis* complex, in the last decade, numerous outbreaks of oriental theileriosis have been recorded in both dairy and beef cattle in the Asia-Pacific region, mainly in Australia and New Zealand (Izzo et al. 2010; Aparna et al. 2011; Islam et al. 2011; Kamau et al. 2011a, b; McFadden et al. 2011; Perera et al. 2013, 2014, 2015c) and, recently, in the USA (Oakes et al. 2019). Based on the characterisation of *T. orientalis* genotypes from cattle affected by clinical oriental theileriosis, only two of the 11 currently known genotypes, namely *chitose* and *ikeda*, are known to be pathogenic, and these two genotypes have been found to be associated with hundreds of recent oriental theileriosis outbreaks in Australasia and the USA (Izzo et al. 2010; Islam et al. 2011; McFadden et al. 2011; Perera et al. 2014, 2015c). Most oriental theileriosis outbreaks occurred when cows had been stressed and immunosuppressed due to calving or late pregnancy and/or other associated farm management, nutritional and environmental challenges (reviewed by Watts et al. 2016).

The main economic losses due to oriental theileriosis relate to abortions, significant reduction in milk production (quantity and quality) in dairy cattle, and severe morbidity and mortality rates in affected cattle herds (Perera et al. 2014; Lane et al. 2015).

Diagnosis of oriental theileriosis and genetic characterisation of members of the *T. orientalis* complex

Various methods have been used for the detection, identification, quantitation and characterisation of *T. orientalis* complex and closely related haemoparasites that infect bovines. The diagnostic methods used for *T. orientalis* can be broadly categorised into traditional and molecular methods.

Traditional methods

The traditional diagnostic methods used for ***T. orientalis*** are mainly based on the clinical signs observed in infected cattle, as well as findings of postmortem, microscopic and serological evaluations. Although traditional diagnostic methods might lack sensitivity and specificity, they are still widely used and useful, to support clinical and epidemiological investigations.

Clinical diagnosis Clinical and postmortem examinations assist the diagnosis of oriental theileriosis. Table 1 provides details of the major clinical signs of this disease. The main clinical signs include anorexia, lethargy, pyrexia, haemolytic anaemia, jaundice, pale mucous membranes, haemoglobinuria, increased heart and respiratory rates, nasal discharge, swollen lymph nodes, depression, abortions, reduced production, still birth and death in severe cases (Islam et al. 2011; Izzo et al. 2010; Aparna et al. 2011; McFadden et al. 2011; Eamens et al. 2013a, b; Perera et al. 2014). As a diagnostic method, this approach can provide some information about the disease occurrence in affected herds. Similarly, postmortem examination of suspected cases can also assist in diagnosis. Gross pathological changes observed in oriental theileriosis include jaundice, pale, rounded and enlarged liver, kidney and spleen, haemorrhagic duodenitis, ulcers in the abomasal mucosa, pulmonary oedema and enteritis (Izzo et al. 2010; Aparna et al. 2011; McFadden et al. 2011; Gebrekidan et al. 2015, 2017a). However, both clinical signs and postmortem findings are often subjective, and are not specific to oriental theileriosis, as diseases caused by other blood parasites can cause similar clinical signs or lesions in bovines (Gharbi et al. 2006; Magona et al. 2008).

Microscopic detection of *T. orientalis* The microscopic examination involves the identification of the parasite (piroplasm) in the red blood cells on Giemsa-stained blood smears (Fig. 1) (Aktas et al. 2006a; Khattak et al. 2012; Perera et al. 2013; Chauhan et al. 2015). Microscopy can also be used to estimate the parasite burden (parasitaemia), particularly in clinical cases when a large number of erythrocytes are infected with *T. orientalis* (see Rogers and Callow 1966; Izzo et al. 2010). However, blood smears are not useful when the parasitaemia is low. Furthermore, it does not allow the discrimination of

Table 1 Clinical signs associated with oriental theileriosis

Geographical location/region	Clinical signs	References
Australia (Queensland)	Pyrexia (> 40.6 °C), anaemia, anorexia, haemoglobinuria, increased heart (120/min), respiratory rates (30/min)	Rogers and Callow (1966)
Australia (New South Wales)	Pyrexia (> 40.6 °C), regenerative anaemia or pale mucous membranes, jaundice, lethargy, tachycardia, tachypnoea, hyperbilirubinaemia, abortions, still births	Izzo et al. (2010), Eamens et al. (2013a, b)
Australia (Victoria)	Pyrexia, regenerative haemolytic anaemia, hyperglobulinaemia, pale mucous membranes, anorexia, neutropenia, leucocytopenia, tachycardia, tachypnoea, abortions	Islam et al. (2011)
India	Pyrexia (40.6 °C), haemoglobinuria, pale conjunctival mucosa, lacrimation, nasal discharge, swollen lymph nodes	Aparna et al. (2011)
Japan	Fever, anaemia, anorexia	Shimizu et al. (1992)
New Zealand	Haemolytic anaemia, pale mucous membranes, anorexia, depression, lethargy	McFadden et al. (2011)
USA	Weakness, icterus, and anaemia (packed cell volume 12.0%)	Oakes et al. (2019)

T. orientalis genotypes or other *Theileria* spp. infecting bovines based on their morphological characteristics. Many *Theileria* spp. infecting bovines are very similar in their morphology at the piroplasm and schizont stages, except for *T. velifera* and *T. taurotragi* which have a unique veil and/or bar-like structures in the infected red blood cells. The main limitations of microscopic examination for *T. orientalis* include lower specificity, sensitivity, inability to identify *T. orientalis* to the genotypic level, and the time required to screen blood smears (Aktas et al. 2006b; Izzo et al. 2010; Aparna et al. 2011; Nayel et al. 2012; Perera et al. 2013; Charaya et al. 2016).

Serological methods The immunofluorescence antibody test (IFAT), enzyme-linked immunosorbent assay (ELISA) and latex agglutination test have been employed for the detection of anti-*T. orientalis* serum antibodies in bovines (Table 2). All these serological tests are targeted at the surface proteins of piroplasms of the parasite(s). Of all available serological methods, ELISA is a relatively sensitive technique to study the prevalence of *T. orientalis* infections in cattle populations (not on an individual animal basis). Serological methods are reported to be more sensitive compared to other traditional diagnostic methods (clinical signs, postmortem and microscopic examinations) used for *T. orientalis*. However, these techniques also have limitations. For example, they are unable to discriminate among different genotypes of *T. orientalis* or differentiate between current and past *T. orientalis* infections, and cross-reactivity with other *Theileria* spp. is common in serological techniques (Mans et al. 2015). Hence, none of the traditional methods used for *T. orientalis* detection allows the differentiation of known genotypes, or yields genetic information about the parasite(s) present. Thus, molecular methods are crucial to identify and distinguish pathogenic and non-pathogenic genotypes of *T. orientalis*.

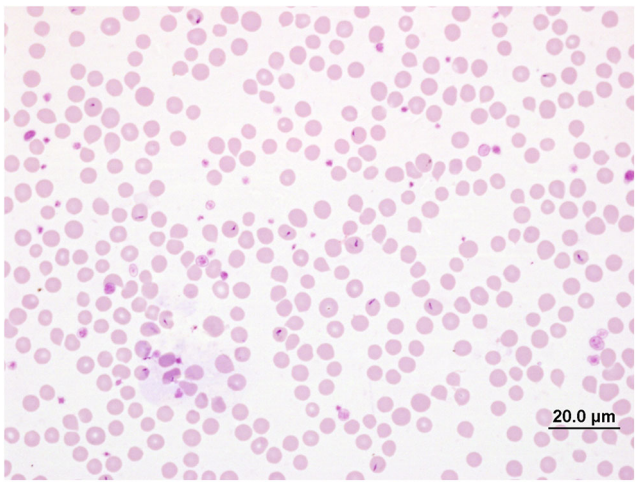

Fig. 1 Giemsa-stained blood smear showing piroplasms of *Theileria orientalis* in red blood cells of cattle. Photo credit, Dr. Natalie Courtman, Melbourne Veterinary School

Molecular detection and characterisation of *T. orientalis*

Molecular diagnostic methods can overcome many of the limitations of traditional methods (Conraths and Schares 2006). To date, a number of molecular assays have been utilised to detect, characterise, differentiate and quantitate members of the *T. orientalis* complex, including conventional polymerase chain reaction (PCR), nested-PCR, reverse line blot hybridisation assay (RLB), loop-mediated isothermal amplification (LAMP), real-time/quantitative PCR (qPCR) using hydrolysis probes and multiplexed tandem PCR (MT-PCR) assays (Table 2).

cPCR This technique is widely used to detect and characterise *T. orientalis*. As compared to traditional diagnostic methods used for *T. orientalis*, conventional polymerase chain reaction (cPCR) has been shown to achieve higher diagnostic sensitivity (DSe)

Table 2 Main methods used for the detection and characterisation of *Theileria orientalis*

Methods	References
Serological	
Indirect fluorescent antibody test (IFAT) using piroplasm antigens	Uilenberg et al. (1985), Papadopoulos et al. (1996)
Enzyme-linked immunosorbent assay (ELISA) using piroplasm antigens	Shimizu et al. (1988)
Latex agglutination test using p33 antigens	Jeong et al. (2005)
Indirect ELISA using p33 antigens	Wang et al. (2010a)
Molecular	
Reverse line blot hybridisation (RLB) using 18S rRNA gene	Gubbels et al. (1999)
Pan-FRET real-time PCR using *cox3* gene	Chaisi et al. (2013)
Conventional PCR using markers *MPSP*, *p32*, *p33*, *p34* and/or *p23*, 18S rRNA (SSU) gene	Tanaka et al. (1993), Kawazu et al. (1995), Kubota et al. (1996), Govaerts et al. (2002), Sarataphan et al. (1999, 2003), Liu et al. (2010, 2011), Ota et al. (2009), Islam et al. (2011)
Semi-nested PCR for 18S rRNA	Ghaemi et al. (2012)
Nested PCR for ITS-1-5.8S-ITS-2 region	Aktas et al. (2007)
Loop-mediated isothermal amplification (LAMP) for *p33* and ITS1-5.8S-ITS2 region	Wang et al. (2010b), Liu et al. (2013)
Quantitative PCR using ITS-1 and ITS-2 regions	Kamau et al. (2011b)
Multiplexed tandem PCR using *MPSP*, ITS-11 and *p23*	Perera et al. (2015a)
Hydrolysis probe-based quantitative PCR using *MPSP*	Bogema et al. (2015)
Multiplexed tandem PCR using *MPSP*	Gebrekidan et al. (2018)

and diagnostic specificity (DSp) using different genetic markers (Perera et al. 2013; Gebrekidan et al. 2016, 2017a, b, c, d; Mans et al. 2015). For this assay, markers including the SSU, *MPSP*, *p23*, 32-kDa piroplasm membrane protein (*p32*), 33-kDa piroplasm membrane protein (*p33*), 34-kDa piroplasm membrane protein (*p34*), COX-III, β-tubulin genes, or the ITS have been used to detect, differentiate and characterise *T. orientalis* from different countries (reviewed by Mans et al. 2015). Of these markers, the *MPSP* gene is the most commonly applied to the *T. orientalis* complex. The cPCR using *MPSP* offers the advantage of amplifying relatively long DNA regions from *T. orientalis*, particularly to characterise novel species/genotypes for the first time compared with established qPCR assays, which usually only amplify short DNA fragments (~ 70–200 base pairs). Limitations of cPCR include its (i) inability to differentiate genotypes in the case of mixed infections with *T. orientalis* and, (ii) potentially, PCR inhibition due to the presence of different blood constitutes such as haemoglobin and lactoferrin, and other chemicals and reagents used for DNA extraction (Perera et al. 2015a). Furthermore, compared with established qPCR assays used for the detection of *T. orientalis* (Perera et al. 2015a), cPCR is less sensitive. In addition, in the cPCR assay, post-PCR steps like gel preparation, gel electrophoresis and purification of PCR products for sequencing are costly, laborious and time-consuming. Based on the molecular size of the PCR products on regular agarose gels, it is difficult to discriminate among genotypes of *T. orientalis* complex. To circumvent this issue, mutation scanning technique, such as single-strand conformation polymorphism (SSCP), has been used to discriminate complex and closely related sequences (from a distinct parasite) even that differ only by a single nucleotide over < 500 bp (cf. Gasser et al. 2006). SSCP has also been used to identify sequence variation within and among genotypes of *T. orientalis* (see Cufos et al. 2012; Perera et al. 2013). The sensitivity of detection of nucleotide differences depends on the size of PCR products and SSCP conditions (Gasser et al. 2006).

nPCR Members of the *Theileria orientalis* complex have been detected in cattle blood samples in Brazil, Iran, South Africa, Uganda and the USA using semi-nested or nested PCR (nPCR) assay employing the SSU or ITS loci (Chae et al. 1998; Oura et al. 2011; Ghaemi et al. 2012; Chaisi et al. 2014; Silveira et al. 2016). The principle of nPCR is similar to that of cPCR, except nPCR requires two rounds of amplification of the marker(s) using outer and inner primers. The inner primers bind to sequences in the target DNA that are within the DNA fragment amplified by the outer primers. The advantage of this technique is that, if the outer primers bind and amplify non-target regions of the DNA sequence, it is rare that the inner primers will bind within the incorrect regions of the DNA (Odongo et al. 2010).

A limitation of nPCR can be the higher probability of contamination as the amplicons of the first round of PCR are diluted for the second PCR, and the chance to contaminate the reaction is high which may lead to false-positive results (Cox-Singh et al. 2000). Furthermore, nPCR requires more time (due to the two rounds of amplification) than cPCR (Janardhanan et al. 2014). The estimation of DSe and DSp of nPCR assay is difficult. However, some studies have compared the estimated DSe and DSp of nPCR and with those of cPCR assays, and found that the DSe of the nPCR was higher than that of cPCR. However, due to the higher probability of false-positive results, DSp of nPCR can be lower than that of cPCR (Kim et al. 2008, 2011; Rigotto et al. 2005).

RLB hybridisation RLB hybridisation allows the detection of multiple molecular targets in a sample using one multiplex PCR reaction, followed by probe hybridisation on a nylon membrane. This technique has been used to screen and differentiate several pathogens of veterinary and medical importance (e.g. Rijpkema et al. 1995; Kamerbeek et al. 1997; Gubbels et al. 1999). The development and validation of a novel RLB hybridisation overcomes most of the limitations of cPCR, which lacks the simultaneous detection of pathogens in the same sample, and it is much more sensitive and cheaper than cPCR. In this assay, multiple pathogens can be detected using multiple probes for simultaneous detection. RLB hybridisation was originally developed for the identification of *Streptococcus* serotypes (Kaufhold et al. 1994). Subsequently, this assay was also used for the detection and differentiation of pathogens in ticks (e.g. *Borrelia spirochetes*) (Rijpkema et al. 1995), and then rapidly became a standard molecular diagnostic tool for the detection and differentiation of known *Theileria* and *Babesia* spp. infecting bovines. For instance, it has been used for the characterisation of *Babesia divergens* in a human case, and for the discovery of novel *Theileria* and *Babesia* spp. (Gubbels et al. 1999; Centeno-Lima et al. 2003; Nijhof et al. 2003; Schnittger et al. 2004; Oura et al. 2004; Brigido et al. 2004; Tomassone et al. 2012). Gubbels et al. (1999) validated an RLB hybridisation assay for the simultaneous detection and differentiation of *Theileria* and *Babesia* spp. infecting bovines, and it was able to differentiate six and three *Theileria* and *Babesia* spp., respectively. The main limitations of RLB assay include a time-consuming protocol with many steps and its low specificity (Mans et al. 2015).

LAMP LAMP is a simple, sensitive, rapid and cost-effective technique that amplifies different templates, including genomic DNA, heat-treated blood and blood dried on filter papers under isothermal conditions (Notomi et al. 2000; Kuboki et al. 2003; Poon et al. 2006; Thekisoe et al. 2010). This technique is a useful diagnostic method to be employed in laboratories with limited resources. It does not require expensive laboratory equipment, such as PCR thermocyclers and fridges/refrigerators, and the reagents are stable at room temperature (25 to 37 °C), which makes this method appropriate for use in field conditions (Thekisoe et al. 2009). Many studies have reported the utility of the LAMP technique for the rapid detection of both *Theileria* and *Babesia* spp. infecting bovines (Iseki et al. 2007; Salih et al. 2008; He et al. 2009; Thekisoe et al. 2010; Liu et al. 2012). However, only two studies have reported the use of LAMP for the diagnosis of *T. orientalis* infection in China (Wang et al. 2010; Liu et al. 2013). One of the main limitations of LAMP for *T. orientalis* is the challenge of designing reliable primers for closely related genotypes (Torres et al. 2011).

qPCR This technique was first described in the mid-1990s, and its popularity and usage grew in the field of molecular microbiology, as it allowed sensitive, accurate and reproducible detection of pathogens in a variety of samples (Bretagne et al. 2001; Nicolas et al. 2002; Bossolasco et al. 2003; Mary et al. 2004; Vitale et al. 2004). It is a reliable assay to measure gene expression, to quantitate parasite intensity and to undertake single nucleotide polymorphism (SNP) analysis. As compared with other commonly used molecular diagnostic tools, such as cPCR, nPCR, RLB hybridisation and LAMP assays, qPCR is more sensitive and less time-consuming, and, once critically standardised and validated, does not require post-PCR analyses, such as gel electrophoresis and DNA sequencing (Francino et al. 2006; Abda et al. 2011). Another advantage of this assay is that high-resolution melt (HRM) curve analysis can be used to infer variation in PCR products. High-resolution melt curve analysis is a rapid, practical and inexpensive technique to discriminate parasites to the species-level based on the melting characteristics of amplicons (Morick et al. 2009; Mehta et al. 2013).

Many studies have evaluated qPCR assay for the detection, identification and quantitation of *Theileria* spp. infecting bovines globally (Sibeko et al. 2008; Papli et al. 2011; Ros-García et al. 2012; Chaisi et al. 2013). Recently, TaqMan qPCR assays were developed for *T. orientalis* genotypes, targeting the *MPSP* gene for use in Australia (Bogema et al. 2015) and in New Zealand (Pulford et al. 2015). These two assays performed similarly, in terms of specificity and sensitivity, but these assays are not able to detect all currently known genotypes of *T. orientalis* or novel genotypes in Australia and New Zealand. Moreover, initial costs linked to the setting up and standardisation of qPCR can be relatively high (e.g., Mackay et al. 2002). Due to the high analytical sensitivity of qPCR assays, sound experimental design, the inclusion of suitable internal controls and data normalisation are crucial to achieving reliable results (Wong and Medrano 2005).

MT-PCR Various duplex and/or multiplex qPCR assays have been developed for the simultaneous detection of different pathogen species, including *Theileria* spp. of bovines (Criado-Fornelio et al. 2009; Peleg et al. 2010; Bilgiç et al. 2013; Junlong et al. 2015). An MT-PCR assay was developed for the simultaneous detection of the four common genotypes (*buffeli*, *chitose*, *ikeda* and *type 5*) of the *T. orientalis* complex known to occur in Australasia (Perera et al. 2015a). This assay used multiple markers, including the *MPSP* gene for genotypes *chitose* and *type* 5, the *p23* gene for *buffeli* and ITS-1 for *ikeda* (Perera et al. 2015a). However, subsequently, Gebrekidan et al. (2017d) detected cross-amplification of *p23* gene and ITS-1 from both genotypes *buffeli* and *ikeda*. Consequently, the MT-PCR assay was modified to detect the four common genotypes (*buffeli*, *chitose*, *ikeda* and *type 5*) of *T. orientalis* by using only one marker (i.e. *MPSP*) in the assay (Gebrekidan et al. 2018). This technique requires the Easy-Plex platform (AusDiagnostics Pty. Ltd., Australia), which includes a 24 well-variant of the Easy-Plex Processor, a 96 well-Easy-Plex Analyser, personal computer with Easy-Plex software, vortexer, 96-well plates, dilution plate, plate sealing films and a liquid handling robot. The assay involves primary amplification using primers designed to each of the genotype of *T. orientalis* targeted, and secondary amplification for quantification using nested primers to internal regions of specific markers used. Samples tested in this assay are recorded as test-positive or test-negative using the auto-call function in the Easy-Plex software. Similarly, the intensity of infection (DNA-copy numbers) for each genotype in each sample is determined by comparison with the cycle threshold data predetermined for an internal spike-control containing a known number of DNA copies (10,000) provided by the AusDiagnostics Pty. Ltd., Australia (cf. Perera et al. 2015a). Although this assay is a time- and cost-effective and sensitive method to simultaneously detect multiple genotypes of *T. orientalis*, it requires specific reagents and equipment, and, thus, can be relatively costly to set up initially.

Considerations regarding individual-animal versus herd-level testing

Although the currently available advanced molecular diagnostic tools used for *T. orientalis* allow for a rapid and accurate diagnosis (mainly for the two pathogenic genotypes *chitose* and *ikeda*), some assays can be expensive for routine use due to individual testing of blood samples, particularly when outbreaks of oriental theileriosis occur in cattle herds. However, herd-level prevalence and intensity of infection with *T. orientalis* can be readily determined at a considerably low cost by testing pooled blood samples from herds. Several studies have evaluated and reported the usefulness and cost-effectiveness of pooled sampling technique for distinct applications. For example, pooled testing has been utilised for the screening of blood samples for malaria from blood donors, the detection of bovine leukaemia and viral diarrhoea pathogens in cattle and *Salmonella* infections in poultry (Muñoz-Zanzi et al. 2000; Singer et al. 2006; Bharti et al. 2009; Hsiang et al. 2010). Recently, the performance and cost-effectiveness of MT-PCR were assessed for the diagnosis of the two pathogenic genotypes (*ikeda* and *chitose*) of *T. orientalis* using pooled blood samples from cattle (Gebrekidan et al. 2017a, b, c, d). Although shown to be useful in this proof of concept study, further experimental optimisation is required before this assay can be routinely used in the monitoring of *T. orientalis* infection(s) in bovine herds.

Conclusions and future implications

Recent outbreaks of oriental theileriosis in cattle in the Asia-Pacific region and the USA have challenged the dogma that oriental theileriosis is a benign infection. Evidence shows that the clinical form of oriental theileriosis can lead to significant economic losses in dairy and beef cattle. Furthermore, the two genotypes (*ikeda* and *chitose*) of *T. orientalis* are mainly linked to the clinical form of oriental theileriosis. The taxonomy and nomenclature of members of the *T. orientalis* complex are still unresolved, and a number of molecular markers have been utilised to characterise *T. orientalis* from cattle. Various aspects of oriental theileriosis such as transmission, pathogenesis, epidemiology and control remain to be fully explored.

A critical appraisal of the literature shows that the currently available traditional diagnostic methods used for the detection of *T. orientalis* can have limitations, particularly regarding diagnostic and analytical sensitivity and specificity. However, most of the limitations associated with the traditional diagnostic methods used for detection can be overcome by using molecular diagnostic tools. Of the currently available molecular diagnostic tools, cPCR employing the *MPSP* gene is the most commonly used technique for the characterisation of *T. orientalis* genotypes globally, although other molecular markers, such as 18S rRNA, ITS and *p23* gene, have also been employed. A few quantitative PCR assays employing different markers have also been developed and validated in Australia and New Zealand to detect, differentiate and quantitate pathogenic genotypes (*ikeda* and *chitose*) of *T. orientalis* (e.g. Bogema et al. 2015; Perera et al. 2015a; Gebrekidan et al. 2018).

Currently available, advanced molecular tools for the diagnosis and characterisation of *T. orientalis* are unable to simultaneously reliably detect/distinguish all of the currently known and/or novel genotypes of *T. orientalis*. Therefore, validation of other molecular tools that can simultaneously detect all currently known and/or novel genotypes of *T. orientalis* is required. It is expected that next-generation sequencing

(NGS) of PCR amplicons using the Illumina platform will be a suitable method that could allow both infection intensity and genetic diversity within *T. orientalis* populations to be measured directly. Recently, Chaudhry et al. (2019) used 18S rDNA spanning V4 hyper-variable region (~500 base pairs) of haemoprotozoa to detect eight and six species of *Theileria* and *Babesia* in cattle and buffaloes in Pakistan. Importantly, some of these species were detected for the first time in Pakistan. A similar approach using the *MPSP* marker could be utilised to detect and quantitate *T. orientalis* genotypes. Such a NGS-bioinformatic approach could help shed light on the biology, epidemiology and population genetics of members of the *T. orientalis* complex.

Funding information This project was supported by Collaborative Research and Early Career Researchers grants (the University of Melbourne) and Research Initiatives Fund grant (the Faculty of Veterinary and Agricultural Sciences, the University of Melbourne) (A.J.). H.G. and A.G. are grateful recipients of the Melbourne International Research Scholarships (MIRS) and Melbourne International Fee Remission Scholarships (MIFRS) through The University of Melbourne. P.K.P received the International Postgraduate Research Scholarship (IPRS) and Australian Postgraduate Award (APA) through the University of Melbourne. Another support was through funds from the Australian Research Council (ARC).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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