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Invited Review

To kill a piroplasm: genetic technologies to advance drug discovery and target identification in *Babesia*

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ABSTRACT

Babesia parasites infect a diverse range of vertebrate hosts, from penguins to pigs. Recently, the emergence of zoonotic *Babesia* infection has been increasing, and the list of species reported to infect humans continues to grow. Babesiosis represents a burgeoning veterinary and medical threat, and the need for novel therapeutic drugs to effectively target this diverse group of parasites is pressing. Here, we review the current culture systems that exist to study and manipulate *Babesia* parasites, and identify the scope and methods for target discovery and validation to identify novel, potent anti-babesial inhibitors. Challenges exist including difficulties in the culture systems of important zoonotic parasites, and there is a lack of integrated morphological and molecular data. While molecular approaches in several *Babesia* spp. has become a reality, the ability to rapidly identify and validate drug targets is hindered by a lack of sophisticated genetic tools to probe parasite biology. The minimal genome size and haploid nature of blood-stage *Babesia* parasites presents an opportunity to adapt techniques from related systems and characterise the druggable genomic space in a high-throughput way. The considerable diversity of parasites within the genus suggests the existence of highly divergent biology and polymorphism that could present a formidable barrier to the development of a pan-babesiacidal therapeutic strategy.

1. Introduction

The genus *Babesia* contains an incredibly diverse group of piroplasmid organisms (Fig. 1). *Babesia* is one of the most common blood-borne parasites in vertebrate animals, second only to the trypanosomes (Vannier et al., 2008). To date, over 100 species of *Babesia* have been described, as well as many more related piroplasmid organisms such as *Theileria* spp. (Vannier et al., 2008). Babesiosis is a significant cause of veterinary disease from canines to cattle (Bock et al., 2004; Solano-Gallego et al., 2016; Eichenberger et al., 2017). More recently, zoonotic infection has been emerging in humans. Human babesiosis is caused by an increasingly diverse array of parasites including *Babesia microti*, *Babesia duncani*, *Babesia divergens*, *Babesia venatorum*, *Babesia crassa*-like and many related, undescribed parasites (Conrad et al., 2006; Bloch et al., 2012; Yabsley and Shock, 2013; Ord and Lobo, 2015; Rajkumari, 2015; Vannier et al., 2015; Jia et al., 2018) (Fig. 1). Indeed, infections in humans have been described from *Babesia odocoilei*-like, *B. divergens*-like, and *B. microti*-like parasites. The recent detection of these new pathogens highlights the potential for emerging infections and the wide-spread nature of the parasite (Herwaldt et al., 1996, 2003; Holman et al., 2005). The emergence of a diverse set of zoonotic parasites, compounded with the abundance of veterinary piroplasmids, presents a unique opportunity to identify conserved biology which can be exploited to identify novel pan-piroplasmid compounds. However, the five recognised clades of *Babesia* parasites display unique biology between them (Schreeg et al., 2016). This poses a challenge in identification of conserved biological mechanisms which can be targeted with small molecules, due to divergence within and between species. This is exemplified by the variation in efficacy of different compounds identified in screening multiple species with the Medicines for Malaria Ventures Malaria Box (Rizk et al., 2015; Hostettler et al., 2016; Paul et al., 2016; Van Voorhis et al., 2016). This poses a challenge in identification of conserved biological mechanisms which can be targeted with small molecules due to the likely existence of high polymorphism within and between species. Conversely, the smaller genome sizes of *Babesia* spp., together with high-throughput genomics, facilitates the identification of core apicomplexan biology which can be exploited for therapeutic development through comparative approaches.

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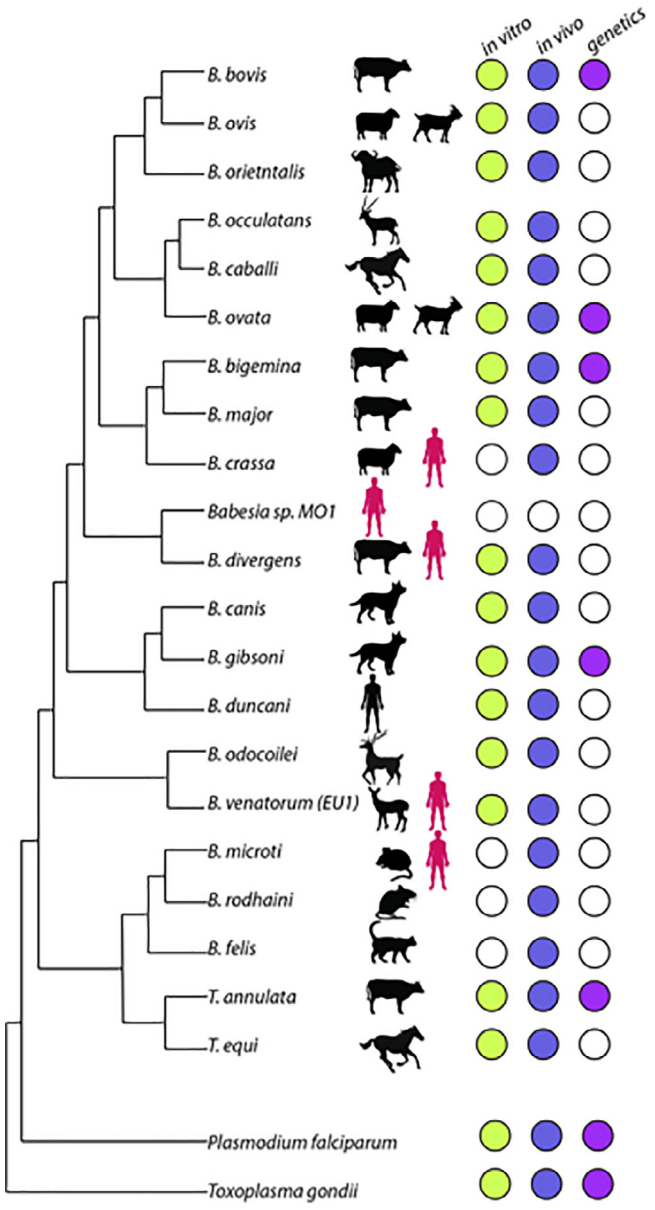

Fig. 1. Presented is an illustrative phylogeny based loosely on available 18S rRNA sequence data and current phylogenetic studies of piroplasm species of veterinary and medical importance, as well as all species which can currently be cultured in vitro. Filled circles represent the existence of the denoted system (green: in vitro culture; blue: in vivo model; purple: genetic system established). Parasites of zoonotic importance are denoted by a red human silhouette. *B.*, *Babesia*; *T.*, *Theileria*.

2. Cultivation systems for piroplasmids of veterinary and zoonotic importance

A major advantage of piroplasmid diversity is the abundance of experimental systems which can facilitate translational discoveries. Despite recent efforts to describe *Babesia* spp. using microscopy, much remains to be understood about the life cycle, including the molecular progression of development and differentiation between sexual and asexual stages (Park et al., 2015; Cursino-Santos et al., 2016). Such discoveries, using the various culture systems discussed, will facilitate future therapeutic development. In vitro culture systems enable drug discovery by increasing the throughput of screening and ease of experimental systems for understanding the biology of these organisms (Astashkina et al., 2012). Many species from the various piroplasmid clades can be propagated in vitro. Indeed, many of the most relevant veterinary parasites including *Babesia bovis*, *Babesia bigemina*, *B. divergens*, *Babesia major*, *Babesia ovata*, *Babesia ovis*, *Babesia gibsoni*, *Babesia canis*, *Babesia caballi*, *Theileria equi* (*Babesia equi*) and *Theileria annulata* can be cultured in vitro (Thomson and Fantham, 1914; Irvin et al., 1979; Levy and Ristic, 1980; Molinar et al., 1982; Vayrynen and Tuomi, 1982; Vega et al., 1985a, 1985b; Goff and Yunker, 1986, 1988; Ben Musa and Phillips, 1991; Holman et al., 1994a; Igarashi et al., 1994; Zweygarth et al., 1995, 1999; Van Niekerk and Zweygarth, 1996; Grande et al., 1997; Viseras et al., 1997; Posnett et al., 1998; Yamasaki et al., 2000; Zweygarth and Lopez-Rebollar, 2000; Musa and Abdel Gawad, 2004; Adaszek and Winiarczyk, 2011; Gharbi et al., 2012; de Rezende et al., 2015) (Fig. 1). Additionally, other animal and wildlife *Babesia* parasite in vitro culture systems have been developed for a few undescribed species, as well as *B. odocoilei*, *Babesia occultans* and *Babesia orientalis* (Holman et al., 1988, 1994b,c, 2005; Thomford et al., 1993; Van Niekerk and Zweygarth, 1996; Zhao et al., 2002) (Fig. 1). While many of these culture systems remain to be fully optimised and may be limited by the appropriate blood source etc., the methods of culture for many species of *Babesia* parasites are relatively simple, and conducive to high-throughput experimentation (reviewed in Schuster, 2002). The existence of such a broad and diverse set of cultivable *Babesia* parasites offers a unique opportunity to explore conserved, essential biology through parallel experimentation in multiple species.

Unfortunately, despite major effort, most human zoonotic parasites cannot be continuously cultured in vitro. However, the WA-1 strain of *B. duncani* has been reported to be maintained continuously in culture (Thomford et al., 1994). Additionally, in vitro and in vivo (i.e. hamsters) models exist to cultivate the rare zoonotic parasite *B. divergens* and related species (Irvin et al., 1979; Vayrynen and Tuomi, 1982; Ben Musa and Phillips, 1991; Grande et al., 1997; Musa and Abdel Gawad, 2004; Holman et al., 2005). Of the more recently identified zoonoses, *B. venatorum* and *B. crassa*, an in vitro culture system has only been described for the former (Bonnet et al., 2009). Recently, an in vitro culture system for *B. microti*, the major etiological agent of human babesiosis, was patented by Fuller Laboratories (USA), but exact details of this system remain to be fully disclosed (Fuller, L., 2018. In vitro propagation of *B. microti*, US Patent 20180080004, U.S.A.P., U.S.A.). As such, the main avenue to study *B. microti* (and the related parasite *Babesia rodhaini*) relies on short-term ex vivo culture or in vivo models, which limits the scale of drug discovery efforts and functional validation (Shikano et al., 1995; Lawres et al., 2016; Saito-Ito et al., 2016). However, in vivo models are valuable for understanding physiological dynamics of infection and facilitate downstream drug and vaccine validation and discovery (Gardner and Molyneux, 1987; Penzhorn et al., 2000; Lawres et al., 2016; Saito-Ito et al., 2016) (Fig. 1).

3. Identification of new anti-babesial inhibitors

The existence of model systems allows for the development of high-throughput screening (HTS) to identify novel anti-piroplasmid compounds. The need for novel drugs to treat veterinary and human babesiosis is pressing. Current treatments in humans have been reported to result in de novo resistance generation, and reports of resistance exist for veterinary parasites as well (Yeruham et al., 1985; Sakuma et al., 2009; Wormser et al., 2010; Lemieux et al., 2016; Simon et al., 2017). Additionally, the targets and mechanisms of action of some current treatments are not fully understood. While the targets of atovaquone (*cytB*) and azithromycin (rpl4) are known (Simon et al., 2017), those for imidocarb and others remain unknown (reviewed in Mosqueda et al.

(2012). Recently there has been effort to repurpose many compounds to combat piroplasmid infection such as epoxomicin, artesunate, triclosan, and others (reviewed in Mosqueda et al. (2012). Efforts to repurpose antibiotics such as Draxxin®, enoxacin, and clofazimine have shown promise (Omar et al., 2016; Tuvshintulga et al., 2017; Silva et al., 2018b). Novel anti-babesial compounds have also been identified, such as the endochin-like quinolones and a series of analogues of the antimitotic herbicide trifluralin (Silva et al., 2013; Lawres et al., 2016). While there are several novel anti-babesials of interest emerging, many of their targets are the same as those for currents treatments (i.e. cytochrome bc1 complex for atovaquone and the new endochin-like quinolones) (Kessl et al., 2003; Lawres et al., 2016). In contrast, trifluralin analogues have been shown to target α-tubulin, a novel target, showcasing the strength of such comparative approaches in identifying lead compounds (Silva et al., 2013). Cysteine proteases, lactate dehydrogenase, dihydroorotate dehydrogenase, apicoplast pathways, and kinases have been proposed as novel targets with some evidence that inhibition of these pathways can inhibit parasite growth (Bork et al., 2004; Okubo et al., 2007; AbouLaila et al., 2012; Kamyingkird et al., 2014; Pedroni et al., 2016). However, the need remains to advance research into these targets, and further identify novel druggable targets.

Thanks to the robustness of several in vitro and in vivo culture systems, screening for novel, effective anti-piroplasmid compounds is possible. Small scale screens have been used to test the efficacy of compounds against several species, often in parallel experiments, such as *Babesia felis*, *T. equi* (*B. equi*), *B. caballi*, *B. bovis*, and *B. bigemina* (Penzhorn et al., 2000; Nagai et al., 2003; Okubo et al., 2007; AbouLaila et al., 2012). Recently, fluorescence based tools have been developed which have enabled larger scale screening for anti-piroplasmid compounds both in vitro (*T. equi*, *B. caballi*, *B. bovis*, *B. bigemina*, *B. divergens*) and in vivo (*B. microti*) (Guswanto et al., 2014; Rizk et al., 2015, 2016, 2017). These advances have allowed screening of larger libraries such as the Medicines for Malaria Ventures 'Malaria Box' in several *Babesia* and *Theileria* spp. in vitro, which have identified many novel compounds with higher potency than previously identified lead compounds of interest (Hostettler et al., 2016; Paul et al., 2016). With the ability to perform HTS in piroplasmids, the next major hurdle lies in the elucidation of the mechanism of action. This relies on a combination of phenotypic characterisation and genetic validation. Thus, robust systems for assessment of drug phenotypes and genetic tools to manipulate the parasite are necessary.

3.1. Biochemical and phenotypic assessment as a method to characterise mechanism of action

There are many ways of identifying potent compounds and parasite targets for further hit to lead optimisation to develop novel effective drugs for *Babesia.* Targeted screening approaches such as recombinant protein assays (ie. DHFR, DHODH, Kinases), can determine direct inhibition of the parasite protein (Brobey et al., 1996; Baldwin et al., 2005; Biftu et al., 2005; Qian et al., 2006; Zhang et al., 2006). These assays in *Babesia* would generally rely on identification of orthologous genes in related parasites. These assays have the advantage of being suitable to HTS of millions of compounds and being specific to the target of interest, being able to counter screen against the host homologs and aid structure based design. However, compounds discovered by this process have several limitations. Firstly, the ability to kill the parasite is dependent on complex properties such as membrane permeability, which is not addressed in these screens. Secondly, the specificity of the compound within the parasite does not always correlate with that seen with recombinant protein. An example of this was with kinase inhibitors, where the activity against recombinant PfCDPK1 protein did not correlate to the anti-*Plasmodium* activity, most likely due to the primary target within the parasite being a different kinase (Ansell et al., 2014). Other widely used methods rely on phenotypic screening, which may help provide a potential pathway that is being inhibited (i.e., apicoplast function, new permeability pathways, calcium signalling, egress, invasion etc.) but generally do not identify a single parasite molecule as the target (Pillai et al., 2010; Salmon et al., 2001; Boyle et al., 2010; Wu et al., 2015; Dickerman et al., 2016; Sidik et al., 2016).

Alternatively, many compounds have been tested in *Babesia* based on their known target in other systems. As described above, many of these compounds have proven to be potent inhibitors of *Babesia*, including two of the most common treatments for human babesiosis, atovaquone and azithromycin. However, the strong reliance of screening compounds that are active against related parasites, mainly *Plasmodium*, will likely not encompass the full chemical space and may miss the most potent anti-babesial compounds. The large evolutionary distance between apicomplexan parasites and more widely studied organisms may lead to false assumptions about a compound's activity and target. This is exemplified by the mTOR inhibitor, Torin 2, which is an extremely potent inhibitor of *Plasmodium falciparum* growth, however, no mTOR homolog is present in *Plasmodium* parasites (Hanson et al., 2013; Sun et al., 2014). More recently, significant advancements have been developed in metabolomics and proteomic methods leading to the identification of several drug-target pairs (Sun et al., 2014; Allman et al., 2016; Creek et al., 2016; Dickerman et al., 2016). While each of these methods are valuable for identifying potential inhibitors and their broad mode of action, genetic methods are still required to validate specific targets within the parasite, and as previously mentioned can be used to identify targets.

A critical step in the characterisation of a novel compound is elucidation of the phenotypic effect generated upon treatment. Stage specificity has been critical in understanding the mechanism of action in the related *Plasmodium* parasites (i.e. Skinner et al., 1996; Sriwilaijareon et al., 2002). In comparison to *Plasmodium*, *Babesia* spp. have a relatively simple asexual life cycle by which parasites invade a red blood cell, egress from the parasitophorous vacuole, grow and divide by binary fission, and finally egress from the host cell (Mackenstedt et al., 1990; Hunfeld et al., 2008; Chauvin et al., 2009; Eisen and Gage, 2009; Mosqueda et al., 2012; Rossouw et al., 2015; Cursino-Santos et al., 2017). For several species such as *B. bovis*, only one division occurs per host cell prior to egress (Hunfeld et al., 2008). However, in other species such as *B. divergens* and *B. microti*, the parasite is able to undergo multiple rounds of binary fission or budding prior to egress (Rossouw et al., 2015; Cursino-Santos et al., 2016, 2017). The differences in asexual cycles between different species present challenges in identifying compounds which have conserved modes of action based on phenotyping alone. However, the unifying developmental steps between all *Babesia* parasites, such as invasion, egress, and motility, provide novel avenues of attack for future compounds. For example, compounds which block egress would lead to an accumulation of merozoites within a host cell, likely regardless of species. Indeed, in *B. bovis* treatment with bumped kinase inhibitors halts egress and leads to an accumulation of merozoites (Pedroni et al., 2016). Similarly, upon treatment with EGTA, *B. divergens* fails to egress and a marked accumulation of merozoites is observed (Cursino-Santos et al., 2017). As egress is a uniquely parasitic process with a clear phenotype, it presents a promising potential target. Compounds which elicit similar or identical phenotypes in multiple species should be prioritised for their pan-piroplasmid potential.

A current challenge in understanding mechanisms of action in a wholistic way is the relative lack of transcriptomic and proteomic

data to characterise the various cellular states of the parasite as it progresses through the cell cycle. Transcriptomic studies that exist have mainly focused on characterizing pathogenesis and host-pathogen interactions (Gohil et al., 2010; Pedroni et al., 2013; Silva et al., 2016a; Eichenberger et al., 2017). Recently, more detailed transcriptomic analyses attempting to characterise intraerythrocytic development and virulence have been released for *B. divergens*, *B. bovis*, and *B. canis* (Pedroni et al., 2013; Rossouw et al., 2015; Eichenberger et al., 2017). The availability of these data provides an important new resource to be used in the identification of novel anti-babesials. Additional detailed transcriptomic studies which fully characterise the cell cycle of both the sexual and asexual stages of *Babesia* parasites will be essential moving forward with high-throughput drug discovery. Proteomic studies have focused on identification of novel secreted antigens, mainly for diagnostic purposes. Smaller studies which study expression of specific families of proteins have been undertaken, but fully characterised proteomes remain to be generated (Alzan et al., 2016; Cornillot et al., 2016; Silva et al., 2016a; Eichenberger et al., 2017; Johnson et al., 2017). Of *Babesia* parasites, *B. bovis* is the most well characterised, and may serve as a model for future technological development (reviewed in Gohil et al. (2010). Compounding these challenges is a relative lack of available complete genome sequences, although culture systems exist for many species of *Babesia*. Genomes exist for *B. microti*, *B. ovata*, *B. bovis*, *B. bigemina*, *B. canis*, and *B. divergens* (Brayton et al., 2007b; Cornillot et al., 2012b; Cuesta et al., 2014a; Jackson et al., 2014b; Eichenberger et al., 2017; Yamagishi et al., 2017). Additionally, genomic sequences for several *Theileria* spp. are available, which could be useful in identifying essential piroplasmid pathways (Gardner et al., 2005; Pain et al., 2005; Hayashida et al., 2012; Kappmeyer et al., 2012).

3.2. Target identification and validation

In order to accelerate drug development, effective means for identifying molecular targets are essential. Due to the ease of phenotypic screening, often potent compounds are identified without a hint as to the mechanism of action. Target identification is important in understanding mechanisms of action and, more broadly, pharmacological properties of a novel compound (reviewed in Schenone et al. (2013). Indeed, target identification is often the first step in the drug discovery pathway. Understanding the target of a compound can aid in understanding potential off target or toxic effects. This helps circumvent costly clinical failures (reviewed in Chan et al. (2010). Simply identifying potential targets is insufficient for the drug discovery pipeline-successful progression of novel compounds relies on both the identification and validation of their interacting partners (Cong et al., 2012). Target discovery and validation can be long and arduous, however for *Babesia* the small genome size (6–14 Mbp) and rapid replication cycle may facilitate this process (Brayton et al., 2007a; Lau, 2009; Cornillot et al., 2012a, 2013; Cuesta et al., 2014b; Jackson et al., 2014a; Eichenberger et al., 2017). The methods by which targets can be identified and validated are subsequently discussed.

3.3. Chemical genomics as a method for target discovery

Using in vitro evolution followed by chemical genomics is an effective method for identifying target-inhibitor pairs in many parasites. This is a strategy whereby resistant organisms are generated against a compound of interest, potential causal mutations are identified through whole genome sequencing, and validated downstream using reverse genetic techniques (Cowell et al., 2018). This strategy was successful in identifying *cytB* as the target of the small molecule inhibitor GNF7686 in *Trypanosoma cruzi* (Khare et al., 2015). In related apicomplexan parasites *Toxoplasma* and *Plasmodium*, chemical genomics is one of the main strategies for target identification (reviewed in McFadden et al. (2001); Luth et al. (2018)). For example, in vitro evolution was used in *Toxoplasma gondii* to identify novel mutations in the dihydrofolate reductase (DHFR) gene which confer resistance to pyrimethamine (Reynolds et al., 2001). Further, this technique has been extensively used in the closely related parasite *P. falciparum* to identify several novel target-inhibitor pairs (Ariey et al., 2014; Corey et al., 2016; Cowell et al., 2018). The existence of many in vitro systems in *Babesia* and *Theileria* presents a unique opportunity to take advantage of chemical genomics in a comparative fashion. Indeed, resistance to several compounds has been generated in vitro in *Babesia*, such as diminazene aceturate in *B. gibsoni* (Hwang et al., 2010). Unfortunately, a genome sequence for *B. gibsoni* remains to be fully elucidated, thus identification of mutations in previously generated lines will be difficult (Goo and Xuan, 2014). Imidocarb dipropionate has been an important treatment for veterinary babesiosis. However, the mechanism of action for this compound has remained elusive (McHardy et al., 1986; Coldham et al., 1995; Rodriguez and Trees, 1996; Belloli et al., 2006; Mosqueda et al., 2012). Drug-adapted lines in *B. bovis* were generated against imidocarb over two decades ago, prior to the genome being published. As the *B. bovis* genome is now available and annotated, sequencing of those isolates may reveal insights into either the mechanism of action or determinants of resistance (Rodriguez and Trees, 1996). Identification of targets for known and novel compounds will be facilitated by the generation of genome sequence of wild-type and previously generated resistant lines in the corresponding species. Once mutations are identified in whole genome sequence, targets will need to be validated. This will require the development of sophisticated genetic tools to probe *Babesia* parasites.

4. Existing genetic technologies in *Babesia* for target validation

The development of genetic tools to study parasites in general has vastly expanded our knowledge of cellular biology and has allowed for the identification and validation of small molecule inhibitor – parasite target pairs: a challenge that remains for *Babesia* spp. Development of transient transfection systems for *B. bovis*, *B. bigemina*, *B. gibsoni* and *B. ovata* has permitted the assessment and optimisation of different transcriptional elements and transfection methods (Suarez et al., 2004, 2006; Suarez and McElwain, 2008; Hakimi et al., 2016; Silva et al., 2016b; Liu et al., 2017a,b). This has since led to the ability to stably transfect each of these organisms, as well as *B. divergens* (Elsworth et al., unpublished data) (Suarez and McElwain, 2009; Hakimi et al., 2016; Liu et al., 2018; Silva et al., 2018a). Using these tools, the generation of parasites with gene deletions, episomal and stable overexpression of two selection markers (BSD and hDHFR) as well as reporter proteins, tick antigens and native *Babesia* proteins for gene complementation, is now possible and has assisted in the elucidation of novel *Babesia* biology (Suarez and McElwain, 2009; Asada et al., 2012, 2015, 2018; Wang et al., 2012; Laughery et al., 2014; Pellé et al., 2015; Hakimi et al., 2016; Oldiges et al., 2016; Alzan et al., 2017; Liu et al., 2018; Silva et al., 2018a; Suarez et al., 2012, 2015).

Many aspects of *Babesia* biology make it highly suitable to genetic manipulation and drug target identification. *Babesia* spp. have a minimalised genome in comparison to most parasites, both in terms of genome size (6–14 MB) and number of genes (~3500–3800) and are also haploid during the asexual cycle, reducing the number of potential targets (Brayton et al., 2007a; Lau, 2009; Cornillot et al., 2012a, 2013; Cuesta et al., 2014b; Jackson et al., 2014a; Eichenberger et al., 2017). *Babesia* spp. have a relatively bal-

anced GC content (~40%) and small intergenic regions – and therefore untranslated regions (UTRs) – aiding in the development of plasmids and sequencing (Brayton et al., 2007a; Lau, 2009; Cornillot et al., 2012a, 2013; Cuesta et al., 2014b; Jackson et al., 2014a; Eichenberger et al., 2017). Homologous recombination is highly efficient and specific, allowing the rapid transfection of linear constructs to manipulate the genome (Suarez et al., 2015). However, the poorly characterised genomes, in terms of gene function and essentiality (with ~50% of the genome having no predicted function), in *Babesia* parasites presents a challenge moving forward (Brayton et al., 2007a; Lau, 2009; Cornillot et al., 2012a, 2013; Cuesta et al., 2014b; Jackson et al., 2014a; Eichenberger et al., 2017). Furthermore, the relative lack of experimental tools and knowledge (synchronisation and purification methods, specific antibodies, genomic, transcriptomic and proteomic datasets, etc.) compared with related parasites presents a major barrier to understanding *Babesia* biology and identifying drug targets, especially in less studied species. It also remains unclear how readily genetic and experimental tools will be transferable between species, with the requirement for generating species-specific reagents likely, and whether compounds will primarily target the same parasite molecule in all species.

5. Looking forward: new genetic approaches for drug target identification in *Babesia*

5.1. Novel genetic tools for understanding gene function

In recent years the genetic toolkit available in parasites has greatly expanded to include methods for rapid and markerless gene editing, inducible knockdown and knockout as well as genome-wide knockdown and knockout studies (a summary of available genetic tools in related organisms is shown in Table 1). Currently no such technologies are available in any *Babesia* species, however, the lessons learnt from related parasites could be used to accelerate the adaptation of such genetic tools.

Currently, the only genetic method for determining gene essentiality in *Babesia* is through attempting gene deletion by homologous recombination. Gene deletion has limited value for studying essential genes as the inability to delete a gene does not strictly show essentiality and provides no information on the function of the gene. For these reasons the parasite field has widely adopted inducible knockdown and knockout strategies (reviewed in de Koning-Ward et al. (2015). Inducible knockdown systems can broadly be grouped into those that alter gene transcription (Tet-off, Cre-Lox) (Gossen et al., 1995; Wirtz and Clayton, 1995; Meissner et al., 2001, 2005; Pino et al., 2012; Collins et al., 2013), mRNA stability/translation (*glmS*, TetR, TetR-DOZI) (Prommana et al., 2013; Goldfless et al., 2014; Ganesan et al., 2016), protein stability (DD and DDD) (Banaszynski et al., 2006; Armstrong and Goldberg, 2007; Iwamoto et al., 2010; Muralidharan et al., 2011; Beck et al., 2014) and protein localisation (knock-sideways) (Robinson et al., 2010; Birnbaum et al., 2017). The appropriateness of each system will depend on the gene of interest (GOI) and intended use. For example, the protein targeting systems require a protein tag that may interfere with the natural function of the GOI. On the other hand, the protein targeting systems tend to act faster (knock-sideways acts within minutes) than the RNA systems, which require natural turnover of the protein (Robinson et al., 2010; Birnbaum et al., 2017). Inducible gene deletion using the Cre-Lox system could also be used and is likely to produce a stronger phenotype than knockdown systems (Andenmatten et al., 2013; Collins et al., 2013). A single study has described the use of RNA interference (RNAi) in *B. bovis*, a technique used in *Trypanosoma brucei* drug discovery, however, the relatively low success rate and lack of identifiable RNAi machinery in the parasite will need to be investigated further before this technique can be widely applied (Baker et al., 2011; Burkard et al., 2011; AbouLaila et al., 2016).

5.2. Genetic methods for drug discovery

Gene editing to introduce point mutations into a GOI can aid in validating the specificity of a compound, for example to validate mutations observed after generation of resistant parasites or through rational mutation based on homologs (Donald et al., 2006; Lourido et al., 2010; Chow et al., 2016; LaMonte et al., 2016; Ng et al., 2016; Crawford et al., 2017; Dhingra et al., 2017; Sonoiki et al., 2017). Parasites harbouring point mutations can be generated by homologous recombination, which is already possible in *Babesia*, or could be generated with a CRISPR/Cas9 system which would require less cumbersome plasmid design (Suarez

Table 1
Genetic tools available for drug target identification. 'Yes' represents technologies that are currently available for that organism. 'Possible' represents technologies where there are no obvious biological features of the organism that would prevent its development. 'Not possible' represents technologies where the biology of the organism is expected to prevent the successful use in that organism.

Genetic tool	Mammalian/ Yeast	*Trypanosoma brucei*	*Toxoplasma gondii*	*Plasmodium falciparum*	*Babesia* spp.
Gene editing					
Homologous recombination	Yes	Yes	Yes	Yes	Yes
CRISPR/Cas9	Yes	Yes	Yes	Yes	Possible
Overexpression					
Stable episomal expression	Yes	Yes	Yes	Yes	Yes
Stable integration	Yes	Yes	Yes	Yes	Yes
Genome-wide overexpression library	Yes	Yes	Possible	Possible	Possible
CRISPR-a	Yes	Possible	Possible	Possible	Possible
Reduced expression					
Conditional transcription	Yes	Yes	Yes	Yes	Possible
Conditional mRNA stability/translation	Yes	Yes	Yes	Yes	Possible
Conditional protein stability/localisation	Yes	Possible	Yes	Yes	Possible
Haploinsufficiency	Yes	Not possible	Not possible	Not possible	Not possible
RNA interference	Yes	Yes	Not possible	Not possible	Not possible
CRISPR-i/Cas13 RNA targeting	Yes	Possible	Possible	Possible	Possible
Gene deletion					
Homologous recombination	Yes	Yes	Yes	Yes	Yes
CRISPR/Cas9-NHEJ	Yes	Not possible	Yes	Not possible	Not possible
Transposon mutagenesis	Yes	Yes	Possible	Yes	Possible

and McElwain, 2009; Hakimi et al., 2016; Liu et al., 2018; Silva et al., 2018a).

Synthetic lethal or overexpression studies, which rely on altered expression of the parasite target causing increased or decreased sensitivity to an inhibitor, respectively, are widely used for target validation or target orientated screening (i.e. Arakaki et al., 2008; Goldfless et al., 2014; Sleebs et al., 2014; Aroonsri et al., 2016). Early *Plasmodium* studies utilised truncated 3′UTRs to reduce the expression level of native genes (Waller et al., 2003; Nkrumah et al., 2009). While this approach could be used with existing techniques in *Babesia*, development of the inducible systems mentioned above would offer a tunable expression system that would be more widely applicable. An alternative approach is to overexpress a GOI, which can either be the native GOI or a divergent homolog with altered susceptibility to a compound, such as *Saccharomyces cerevisiae* DHODH expression in *P. falciparum* (Baldwin et al., 2005; Gardiner et al., 2006; Painter et al., 2007; Ganesan et al., 2011; Hoepfner et al., 2012; Sleebs et al., 2014; Phillips et al., 2015; Dickerman et al., 2016). This can be achieved either by episomal expression of the gene, with the advantage of multiple extra copies, or integration of a second copy of the gene into the genome, which will consistently produce a single extra copy.

5.3. Prospect for forward genetic screens

The technologies available for *Babesia* are best suited to targeted approaches, with either a hypothesised target of a compound or for screening compound libraries against a high priority target of interest. Genome-wide screens that look for genes that are over- or under-represented when exposed to an inhibitor have been widely used in drug discovery with no prior knowledge of the inhibitor's mechanism of action. This can be done with genome-wide overexpression libraries as has been performed in *Leishmania infantum* and *T. brucei*, using methods which could be adapted to *Babesia*, however, it would require significant effort to generate the library (Begolo et al., 2014; Koushik et al., 2014; Gazanion et al., 2016; Tejera Nevado et al., 2016; Fernandez-Prada et al., 2018). To date no large-scale knockdown screens have been performed in parasites lacking RNAi, however, novel high-throughput methods have recently been developed for mammalian cells and have been widely adopted in the mammalian drug screening field (Gilbert et al., 2013, 2014; Konermann et al., 2015; Joung et al., 2017). Cas13 is a newly described molecule that is able to specifically cleave target mRNA, with the only requirement for activity being the presence of a corresponding guide RNA, thus making library production more feasible (Abudayyeh et al., 2016, 2017; Cox et al., 2017; Konermann et al., 2018). The lack of non-homologous end joining (NHEJ) repair mechanisms in *Babesia* precludes the use of CRISPR/Cas9 to perform genome-wide knockout screens, however, the piggyBac transposon, which randomly inserts into TTAA sites in the genome, has recently been used in *P. falciparum*, which can be used to help prioritise essential genes (Zhang et al., 2018). An advantage of the piggyBac system is that insertion into the 5′ and 3′ UTR of genes can lead to altered gene expression (Pradhan et al., 2015; Zhang et al., 2018). Using pools of piggyBac mutants has revealed novel genes that alter sensitivity to inhibitors as well as networks of mutants that have similarly altered sensitivity to multiple compounds, suggesting a related role in the parasite (Pradhan et al., 2015; Zhang et al., 2018).

6. Conclusions

Perhaps the greatest challenge in identifying novel anti-babesials is the extraordinary diversity of the genus. The variability of the *Babesia* genus generates uncertainty about the ease of adapting tools from orthologous systems. Yet, this diversity provides a unique opportunity to study the parasites in a comparative manner to identify core, conserved biology. The large number of piroplasmid species of medical and veterinary importance necessitates the development of a species-transcendent compound. To date, no single compound shows broad range efficacy against all species. Further, no target-inhibitor pairs have been conclusively validated in *Babesia* spp. using genetic techniques. In related species, there has been rapid development of systems for genetic manipulation to identify and validate druggable genes. This has lead to the discovery of lead compounds and accelerated compound development (Phillips et al., 2015, 2016). The plethora of in vitro and in vivo culture systems which are available for piroplasmids offers an unprecedented opportunity to identify novel, conserved biology which could be leveraged in development of species-transcendent compounds for treatment of medical and veterinary infections. The recent success of transfection methods for *Babesia* spp. offers the prospect for development of more advanced techniques. Such techniques would greatly accelerate drug discovery and development.

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