Pursuing effective vaccines against cattle diseases caused by apicomplexan protozoa

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Abstract

Apicomplexan parasites are responsible for important livestock diseases that affect the production of much needed protein resources, and those transmissible to humans pose a public health risk. Vaccines, recognized as a cost-effective and environmentally friendly method for the prevention of infectious diseases in livestock, can avert losses in food production and decrease the exposure of humans to zoonotic pathogens. This review focuses on the need for and advances in vaccine development against the apicomplexan parasites *Theileria* spp., *Babesia* spp., *Toxoplasma gondii*, *Neospora caninum*, *Eimeria* spp., *Besnoitia* spp., *Sarcocystis* spp., and *Cryptosporidium parvum*. Together, the effect of these parasites on the cattle industry worldwide causes an enormous burden, yet they remain poorly controlled and very few effective and practical vaccines against them are available. Vaccine development is hampered by our scarce and limited knowledge of the biology and mechanisms of pathogenesis of these microorganisms, and the absence of correlates of host immune protection. More studies focused on these aspects as well as on the identification of parasite vulnerabilities that can be exploited for vaccine design are needed. Novel "omics" and gene editing approaches in understanding complex parasite biology together with advances in vaccinology will facilitate the development of effective, sustainable, and practical vaccines against cattle diseases caused by apicomplexan parasites. Such vaccines will help prevent animal and human diseases and allow production of enough animal protein to feed the growing human population in the twenty-first century and beyond.

Keywords: cattle, vaccination, animal production, apicomplexan protozoa, *Theileria*, *Babesia*, *Toxoplasma*, *Eimeria*, *Sarcocystis*, *Besnoitia*, *Cryptosporidium*

Review Methodology: For this review study, we performed systematic searches of scientific manuscripts focused on vaccines against cattle diseases caused by apicomplexan protozoa. We searched academic databases, such as PubMed, Scopus, and Web of Science. We searched for terms, such as cattle, *Babesia*, *Theileria, Toxoplasma*, *Neospora*, *Eimeria*, *Besnoitia*, and *Cryptosporidium*, among others. We also used as reference the chapters on *Neospora* and *Eimeria* of the book "Parasitic Protozoa of Farm Animals and Pets" (M. Florin-Christensen & L. Schnittger, eds.), Springer, Cham, Switzerland, 2018 (ISBN 978-3-319-70131-8). Timeframe of our search covered scientific manuscripts from early 1970s to 2021. In addition, institutional websites, such as the European Food Safety Authority and the USDA Animal Research Service, were also consulted in order to gather information on the studied subject.

Introduction

Protozoa are a highly diversified group of single-celled eukaryotic organisms of the kingdom Protista. They can be free-living or parasitic and are distinctive by their animal-like behaviors, including predation and motility. Some protozoa play beneficial roles to their ruminant hosts while living in symbiotic association with their digestive systems. However, some species of parasitic protozoa can be responsible for important and mostly neglected or emerging diseases in livestock, and some of them have potential for zoonotic transmission [1]. Depending on their motility and cell structure, protozoa can be classified as amoebas, flagellates, ciliates, and Sporozoa (syn. Apicomplexa). As the classical name Sporozoa for Apicomplexa suggests, sporozoan parasites have the ability to form sporozoites, a motile form that invades cells in the vertebrate host at some point in their life cycles, initiating an asexual reproductive cycle in their respective vertebrate hosts. In addition, Apicomplexa, as their name in effect implies, are equipped with a set of secretory organelles that form the apical complex and are required for host cell invasion [2].

Some apicomplexan parasites are responsible for diseases of human importance, including malaria, while others have a high global impact on livestock productive systems. Apicomplexan parasites that are responsible for cattle diseases that are addressed in this review belong to the Piroplasmida, Coccidia, and Cryptogregarina taxons [1].

Piroplasmida, such as *Theileria* and *Babesia*, require Ixodid ticks as arthropod vectors that, while feeding, directly inoculate sporozoites into a vertebrate host (inoculative transmission). The dixenous coccidian parasites *Toxoplasma*, *Neospora*, and *Sarcocystis* are transmitted to a ruminant host by ingestion of infective oocysts present in the feces of the infected definitive carnivore host. In turn, the carnivore acquires the parasite after consumption of the infected prey animal (consumptive transmission). The coccidium *Besnoitia* is believed to have a dixenous life cycle, but its mode of transmission is still to be unraveled. In the case of the monoxenous apicomplexans, the coccidium *Eimeria* and the Cryptogregarina *C. parvum,* oocysts excreted with the feces are later taken up orally by the same host species (fecal-oral transmission) [3]. Moreover, transplacental transmission from mother to fetus (vertical transmission) is a main route of infection in the case of the coccidians *Toxoplasma* and *Neospora* and has been also reported for piroplasmids [4–8].

Vaccines are preventive tools aimed at decreasing the severity of illnesses and remain the most cost-effective and environmentally friendly approach to the control of infectious diseases, yet only a few vaccines are currently available to prevent diseases caused by apicomplexan parasites in livestock. There are many reasons for this, including the complexity of their life cycles and their relationships with their hosts, and our incomplete knowledge on the biology of these parasites, mechanisms of pathogenesis, and the nature of the protective immune responses that they elicit. Importantly, parasitic diseases remain essentially neglected and are rarely the focus of investment of large research funds. This might be partly because many parasitic diseases occur in less wealthy and less developed regions of the world. However, the changing dynamics of some of these diseases due, in part, to worldwide climate change and globalization is modifying these perceptions.

This review focuses on the state of the art of vaccines and vaccine development against apicomplexan protozoan parasites responsible for major diseases of cattle. Endemic regions of many of these parasites are quickly expanding, and some, such as *Cryptosporidium* and *Toxoplasma*, have an important direct impact on human health, posing additional public health risks that need to be addressed immediately.

Phylogenetic relationships and basic biology of apicomplexan protozoa

Among apicomplexans of veterinary relevance that cause important diseases in livestock, coccidians (*Toxoplasma*, *Neospora*, *Eimeria*, *Sarcocystis*, and *Besnoitia*) are able to form cysts, and some have a vertebrate carnivore as a definitive host (such as dogs or cats) and are transmitted via feces. Piroplasmids (*Babesia* and *Theileria*) belong, together with *Plasmodium* spp., to Haemosporidia, characterized for infecting erythrocytes as part of their life cycle and the use of an Ixodid tick, as their definitive host [9]. Finally, *Cryptosporidium parvum* is a zoonotic parasite that has recently been reclassified within the Cryptogregarina since it is more closely related to apicomplexan gregarines than to Coccidia and Piroplasmida [10]. The phylogenetic relationships of these parasites are depicted in Fig. 1. It can be observed that the monoxenous *Eimeria* is a sister taxon of dixenous coccidians, whereas *Cryptosporidium* is a sister taxon to all other taxons included in the tree, suggesting a more ancient evolutionary origin.

As members of the phylum Alveolata, apicomplexans are characterized for having flattened vesicles or sacks known as alveoli underlying their cell membrane. They display an obligate parasitic lifestyle and have a great ability to manipulate their hosts. Characteristically, they are equipped with an apical complex, and most contain a unique plastid, known as the apicoplast. The apical complex, which defined and gave the name to this group of parasites, plays a fundamental role in the process of host cell invasion and contains a set of secretory organelles, such as rhoptries, micronemes, and dense granules or spherical bodies, as well as a polar ring and, except for Haemosporidia, a conoid [2]. The apicoplast is a relic of a photosynthetic organelle structure, which originated from a secondary endosymbiotic event involving a red alga and contains a genome of about 30–40 kb. Importantly, plastids are involved in the biosynthesis of fatty acids, isoprenoids, and heme and can be targeted by pharmacological interventions [11]. In contrast to coccidians and piroplasmids, *Cryptosporidium* lacks an apicoplast and mitochondria and can complete its

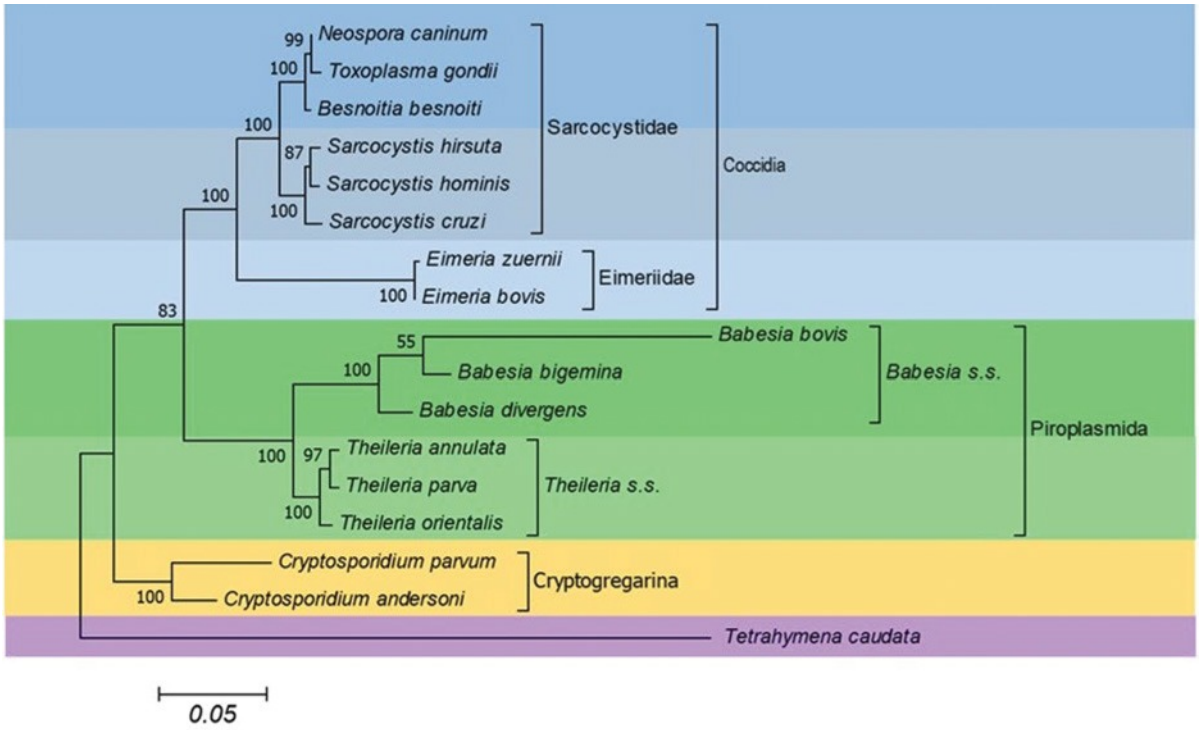

Figure 1. Molecular phylogenetic tree based on 18S rRNA gene sequences of bovine apicomplexan protozoa that infect cattle. After alignment of sequences, the tree was inferred by maximum likelihood, using *Tetrahymena caudata* as outgroup. Accession numbers of sequences are *Neospora caninum*, L24380; *Toxoplasma gondii*, U00458; *Besnoitia besnoiti*, KJ746531; *Sarcocystis cruzi*, KT901167; *Sarcocystis hirsuta*, AF017122; *Sarcocystis hominis*, JX679470; *Eimeria zuernii*, KT184356; *Eimeria bovis*, KT184336; *Babesia divergens*, FJ944825; *Babesia bigemina*, X59604; *Babesia bovis*, HQ264112; *Theileria parva*, L02366; *Theileria annulata*, AY524666; *Theileria orientalis*, AB520954; *Cryptosporidium parvum*, AF164102; and *Cryptosporidium andersoni*, AY954885. The length of the bar corresponds to the number of amino acid substitutions at any given site.

life cycle in host cell–free in vitro cultures, which questions its classification as an obligate intracellular apicomplexan [10, 12].

Apicomplexan parasites typically have complex life cycles that alternate sexual and asexual reproduction. Parasites adopt distinct morphologies and their life cycle stages and multiple antigenic variants move around diverse body or tissue environments in their hosts, including the infectious sporozoite stage, intracellular trophozoites and schizonts, invasive merozoites and ookinetes, and gametes, among others. With the exception of the gametes of some species, apicomplexans do not possess cilia or flagella and move by gliding motility [13]. Except for *Cryptosporidium* and the coccidian *Eimeria*, which have a monoxenic life cycle, all other apicomplexans discussed are dixenous and, thus, require one definitive host where they undergo their sexual mode of replication and at least one nondefinitive or intermediate host where the parasites reproduce asexually. For piroplasmids, the definitive host is an invertebrate, the tick, whereas the intermediate host is a vertebrate, including livestock. On the other hand, the dixenous coccidian parasites addressed in this review use a vertebrate carnivore as a definitive host (Fig. 2). In the case of dixenous coccidia and piroplasmids, sexual meiotic reproduction happens in the definitive host and asexual mitotic propagation in the intermediate vertebrate hosts. In monoxenic apicomplexans, the sexual phase and several cycles of massive asexual propagation happen in a single vertebrate host. With the host feces, either from the only host in monoxenous or from the definitive host in dixenous parasites, an environment-resistant oocyst is excreted in massive quantities, which significantly increases the probability of parasite transmission [3].

For vaccine design purposes, it is of great importance to take into account the characteristics of the biology and life cycle of these parasites, including their antigenic composition, which may differ drastically within a species. Thus, strategies toward developing new vaccines include defining which parasite stage would be more efficiently targeted by a protective immune response. Alternatively, novel vaccines might need to be directed against several distinct life cycle stages in order to be effective.

Current options for the control of cattle diseases

Nowadays, we witness a dramatic expansion of diseases caused by apicomplexan parasites in cattle. This has a direct effect on the production of very much needed protein resources worldwide. In addition to their effects on current

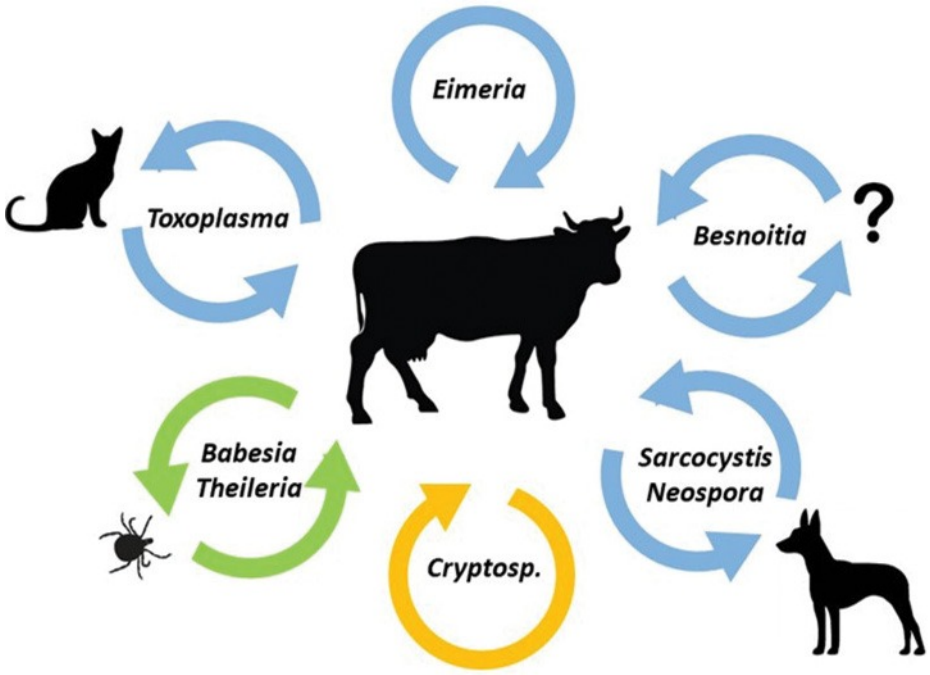

Figure 2. Schematic representation of the life cycles of apicomplexan protozoa that infect cattle. The cycles of monoxenous parasites are depicted with closed arrows, and those of dixenous parasites with open arrows. The respective definitive hosts are represented inside the circles. *Sarcocystis* spp. are exemplified with *S. cruzi*, for which canids are the definitive hosts. The definitive host of *Besnoitia* spp. remains currently unknown. The color of the arrow corresponds to the different phylogenetic groups depicted in Fig. 1.

animal stock, these diseases also impose a severe limit to the introduction of susceptible animals, which is needed to increase milk and meat production in areas where persistent infections occur. This poses an increased risk, as this is becoming common practice in many countries in order to improve animal production. In general, some of the apicomplexan parasites that cause disease in cattle can be controlled using a variety of drugs [14, 15]. However, these treatments are usually costly, may be environmentally toxic, and have the potential to generate undesirable residues that are not allowed in the food chain. In addition, excessive and indiscriminate drug usage often leads to the selection of drug-resistant populations. Other methods of control may rely on the management of cattle, preventive measures, manipulation of the definitive host, and, in the case of piroplasmids, eradication of the tick vector [16].

Vaccines to prevent infections are an environmentally friendly option, but for most of these pathogens, they are yet not available. Reasons for the current lack of vaccines are multiple. In some cases, the diseases are neglected, and little research efforts have been invested. As a result, there are considerable knowledge gaps on the biology of most of these parasites, their relationship with hosts, and the immune mechanisms leading to protection, among others. On the other hand, the development of vaccines is usually difficult due to the complexity of the parasite-host relationships and our inability to culture and genetically manipulate some of these parasites and to decipher the immune mechanisms involved in protection. Furthermore, studies of the immune defense, parasite cultures, and vaccine trials in cattle are expensive and require extensive and costly infrastructure and reagents, for which reason they are out of the reach of the majority of research groups [17]. Importantly, it is recognized that apicomplexan parasites have a particularly long history of coevolutionary interactions with their definitive and intermediate hosts resulting in the development of a large repertoire of mechanisms that allow them to evade the hosts' immune systems. As a result, these parasites can establish persistent infections in the absence of obvious clinical signs, suggesting a compromise of "peaceful coexistence" between the parasite and host. However, these compromises, which lead to inapparent and usually chronic infections and ensure transmission of the parasite to other hosts, are sometimes precarious and may lead to serious disease or other consequences, such as decrease in milk and protein production and abortions. Parasite adaptations due to the long-term parasite-host coevolution, coupled with a large parasite genetic diversity, complicate the development of effective vaccines. During evolution, the parasites already visited, survived, and were selected upon exposure to a large number of possible scenarios presented by their hosts and the environment in their long struggle for survival. In addition, parasite populations are usually complex and genetically diverse and may be composed of several subpopulations with distinct fitness and geno-/phenotypes, some of which can eventually emerge upon selective pressures. Thus, successful parasites may find redundant

pathways to overcome the constraints imposed by the host immune responses and/or may develop different strategies to escape or regulate the host immune system. These realities imply the need of adopting vaccine development strategies that take into account the challenges posed by these complex parasites. On one hand, antibodies against highly antigenic variable antigens might be effective in blocking the parasites, but they may not be effective as vaccine components, whereas on the other hand, conserved functionally relevant antigens might be antigenically subdominant and/or poorly exposed to the immune system effectors. In summary, apicomplexan parasites are master manipulators, may have genomic plasticity, and are adaptable and efficient cell invaders. Therefore, identifying parasite vulnerabilities and life cycle bottlenecks is of great importance for developing efficient methods of control, including vaccines. However, the above-mentioned characteristics of these parasites pose a considerable challenge to vaccine development and can only be overcome by adequate internationally concerted and focused, often multidisciplinary, research efforts.

Effective control methods should be based on parasite-specific targets. The quest for such targets is complicated since parasites and hosts are coevolved eukaryotes and thus share a large number of essential metabolic pathways. On the other hand, reduction of some biochemical pathways has occurred during parasite evolution, with replacement by mechanisms that scavenge metabolites from the host, which has resulted in a reduced number of potential molecular targets. Once a target has been identified, it is highly desirable that parasites are not equipped to circumvent it, that is, the target needs to remain as such in the short and long term. This may be a daunting, but not an impossible, task that requires a deeper knowledge of the biology of the parasites to identify potential "Achilles heels." Another possible risk is the possibility of applying control measures that may result in the selection of parasite variants that may be able to infect novel hosts, where they would be, at least in theory, more pathogenic. On a positive note, the quest for improved drugs and vaccines against apicomplexan parasites will be facilitated by the application of a new arsenal of research strategies that have been recently developed and are now available to researchers, including genomics, proteomics, and other "omics," in conjunction with advances in vaccinology, immunology, and novel genetic manipulation techniques, such as CRISPR/Cas9 and stable transfection [18].

In the following sections, we will address the state of the art on the development of vaccines against the above introduced apicomplexan parasites of cattle.

Current status of available vaccines and vaccine development

Vaccines against Theileria *spp. of importance to cattle*

Tick-borne apicomplexan parasites of the genus *Theileria* cause some of the most economically important diseases that impact the cattle industry worldwide. Here, we focus on relevant studies on vaccine development against *Theileria annulata*, *T. parva*, and *T. orientalis*, three major parasites of this genus and the etiological agents of tropical theileriosis, East Coast fever (ECF), and oriental theileriosis, respectively. During a tick bite, infective sporozoites are transmitted to the cattle host. Clinical disease caused by *T. annulata* and *T. parva* occurs when sporozoites infect leukocytes and develop into macroschizonts, which induces uncontrolled leukocyte proliferation [19]. By contrast, it is the *T. orientalis* piroplasm stage, which infects the host red blood cells, causing anemia that is ultimately associated with the clinical signs of acute oriental theileriosis [20]. Improvement of the currently available vaccines and development of novel, efficient strategies to control *Theileria* parasites are critical to ensure elevated standards of animal health and supply the human population access to high-quality animal protein.

T. annulata infects wild and domestic animals in Southern Europe, Northern Africa, and the Middle East. In addition, India, China, and some Southern locations in Russia are also affected by the parasite [21–23]. It is estimated that more than 250 million cattle are currently at risk of developing an infection with *T. annulata,* which poses a dramatic risk to animal production and food safety in affected areas. The prevalence of tropical theileriosis is difficult to assess in part due to intrinsic differences in susceptibility to the disease of *Bos taurus*, *Bos indicus*, and indigenous breeds of cattle. *T. annulata* and *Theileria* parasites in general present a complex life cycle with development of sexual stages, kinetes and sporozoites in the tick vectors, and schizonts and piroplasms in their vertebrate hosts. *T. annulata* sporozoites are transmitted to cattle by infected ticks upon feeding and rapidly invade MHC class II–expressing cells, mainly monocytes and B cells, which leads to the formation of schizonts and uncontrolled proliferation of the parasite-infected host cells [24, 25]. A percentage of schizonts form merozoites that parasitize erythrocytes and develop to piroplasms, which are eventually acquired by ticks during feeding on an infected host and later transmit the parasite to a naïve bovine. Clinical signs of acute *T. annulata* infection are mainly associated with malignant uncontrolled proliferation of infected leukocytes and, to a lesser extent, to hemolytic anemia caused by the replication of piroplasms in red blood cells [24].

Historically, strategies to control tropical theileriosis have relied on approaches to decrease tick infestation, the use of anti-theilerial drugs, and in some areas the use of indigenous and cross breeds of cattle that are more resistant to the parasite. In addition, the use of attenuated macroschizont cell lines as vaccines has been successfully utilized to control the clinical signs associated with acute *T. annulata* infection [26, 27]. Despite the fact that attenuated cell lines induce protection, concerns remain regarding this strategy. Specifically, vaccination with attenuated cell lines can potentially lead to the development of parasite piroplasms that may be acquired by ticks in the field, which can result in the spread of the infection and,

eventually, to an increase in parasite diversity [28, 29]. Considering these limitations, efforts have been concentrated on the discovery of novel antigens associated with different stages of the parasite for the development of approaches to prevent infection and/or the clinical signs of acute disease. Consequently, several *T. annulata* antigens have been identified, and specifically, two of them, the surface sporozoite antigen 1 (SPAG-1) and the merozoite surface antigen 1 (TAMS-1), have received special attention as potential vaccine antigens [30, 31]. Vaccines based on SPAG-1 and TAMS-1 have been tested using numerous immunization regimes and delivery systems [32–35]. In addition to showing significant levels of protection, results indicate a potential synergistic effect of SPAG-1 and TAMS-1 in inducing protection against *T. annulata* [36]. Despite the promising results, definitive evidence of the efficacy of SPAG-1– and TAMS-1–based vaccines in context with the development of protective cellular immune responses to *T. annulata* remains to be established.

Immunological approaches have also been used as a strategy for the discovery of novel *T. annulata* vaccine antigens. As a result, *T. annulata* antigens 5 (Ta5) and 9 (Ta9) have been identified in context with CD8+ T cells [29, 37]. Interestingly, these studies have shown that the role of cytotoxic cells is less evident in the protection to *T. annulata* than to *T. parva*, as *ex vivo* studies using CD8+ T cells from immune cattle have identified only a few schizont antigens [37]. This suggests that alternative and more efficient strategies are needed to reveal additional, previously unknown, vaccine targets of *T. annulata*. In this context, a recent study proposed the use of a mouse-tick infection model to investigate mechanisms used by *T. orientalis* sporozoites to invade the host leukocytes [38]. However, it remains to be determined if the mouse model represents an improved approach compared to *in vitro* and *in vivo* strategies using bovines for the development of efficient vaccines to control tropical theileriosis.

Another recent study used *in silico* analysis combined with gene expression in tick stages of the parasite to identify *T. annulata* candidate antigens for a transmission-blocking vaccine. By searching the *T. annulata* genome for amino acid domains on similar transmission-blocking sexual-stage targets from *Plasmodium* and *Babesia* parasites, this study identified candidates encoding the 6-cys and cysteine-rich domain protein families [39]. Vaccine trials in cattle are needed to test these newly identified vaccine candidates in their efficiency to block *T. annulata* transmission. Overall, despite recent progress, no subunit vaccine is currently available against *T. annulata*. Therefore, it is important to investigate the profile of antibody and cell immune responses in naturally infected, immune, as well as in vaccinated, animals to identify novel vaccine antigens and biomarkers of protection. In addition, detailed investigation of the parasite life cycle may also reveal bottleneck steps, such as the molecular mechanisms of sexual reproduction that can be potentially exploited for vaccine development.

T. parva causes an acute and usually fatal condition named ECF that affects bovines in Eastern and Southern Africa. The disease poses a tremendous economic impact, especially on pastoralist and small farmers in the affected regions. Following transmission to cattle by infected ticks, parasite sporozoites enter and develop within the cytoplasm of lymphocytes, primarily CD4+ and CD8+ cells. Inside these lymphocytes, sporozoites transform into schizonts that induce uncontrolled proliferation of the infected cells, which, in turn, promotes parasite growth [40]. In addition to the lymphoproliferative aspects of the disease, dysregulation of the host immune responses to *T. parva* has also been associated with the acute signs of ECF [41, 42]. The development of persistent infection in animals that survive the acute phase of the disease and the presence of asymptomatic reservoir animals in affected regions, such as water buffaloes, demonstrate the complexity of the disease epidemiology. In addition, the widespread tick vectors, mainly *Rhipicephalus* ticks, in endemic areas poses an extra challenge for the development of control strategies.

The infection and treatment method (ITM) has been historically the primary choice to prevent the dramatic effects of ECF. This method consists of inoculating animals with a cocktail of *T. parva* strains following administration of long-acting oxytetracycline [29, 43]. Despite inducing a protective immune response, ITM presents several constraints, such as high cost and complexity of the production of vaccine batches and need for a cold chain for distribution. Another downside of ITM is the establishment of persistent infection in vaccinated animals and the potential risk for tick transmission, even though recent studies showed that ITM parasites do not replace the local parasite population, which, at least in part, favors the use of ITM to mitigate acute ECF [44, 45]. In addition, ITM relies on the use of oxytetracycline, which raises concerns on the spread of antibiotic resistance and contamination of food and dairy products. Altogether, these factors indicate that novel and more sustainable vaccines are needed to control ECF. The polymorphic immunodominant molecule (PIM) is a well-characterized protein expressed by both the sporozoite and schizont stages of *T. parva* and has been evaluated as a vaccine antigen using different delivery platforms [46, 47]. Despite eliciting strong humoral and cellular immune responses, PIM vaccination induced no protection. This finding is in agreement with similar observations in vaccine trials using comparable immunodominant antigens in distinct apicomplexan hemoparasites, such as *Babesia* spp. [48]. The *T. parva* p67 is another well-studied antigen expressed on the surface of sporozoites that has been evaluated in subunit and virus-vectored vaccines to control ECF [49, 50]. Although vaccination with p67 induces the development of *in vitro* neutralizing antibodies, variable levels of protection in field trials have been observed following challenge with parasite sporozoites [51, 52]. Explanations for this finding may rely on the fact that other immune

mechanisms, besides antibodies, may be involved in protection induced by p67 and that novel antigen delivery systems may need to be utilized to improve the efficacy of p67-based vaccines [53, 54]. In that context, a recent study reported the use of novel nanotechnologies to present a polypeptide derived from p67 C-terminal to the immune system of cattle [55]. Despite the variation among animals, results showed significant protection by comparing vaccinated and control animals, which encourages further evaluation of this vaccination approach.

It has been established that the development of cell-mediated immune response is associated with protection against ECF [56, 57]. In this regard, a seminal study used adoptive transfer immunity to demonstrate that CD8+ cytotoxic T cells are implicated in controlling the spread of schizont-infected cells and, ultimately, responsible for protection against ECF [56]. In addition, it has been demonstrated that genomic polymorphisms of parasite strains and isolates and MHC haplotype diversity of the vertebrate hosts also influence ECF pathogenesis and immunity [58–61]. Altogether, these aspects have set the rationale to identify novel *T. parva* antigen candidates for vaccines. Recent studies screening CD8+ T-cell lines from *T. parva*–immune cattle have identified promising vaccine candidate antigens, named Tp1-10 and Tp12 [62–64]. In a recent subsequent study, Tp1 antigen expressed in human adenovirus and vaccinia virus induced specific cellular immune response in vaccinated animals [60]. However, only 36% of the Tp1-vaccinated cattle survived a lethal challenge with *T. parva*, suggesting that additional antigens and delivery systems are needed to have an efficient vaccine to elicit protection against ECF. Considering current progresses and shortcomings, further studies are needed to evaluate the potential of these novel Tp antigens in vaccine formulations. Collectively, these studies that used CD8+ T cells to identify novel *T. parva* antigens represent a breakthrough in reverse vaccinology that can be applied to other apicomplexans. Altogether, regarding the availability of ITM as a control strategy to ECF, this method presents several logistical, economic, and scientific drawbacks, and therefore, the development of efficient and more sustainable alternative vaccines is required. We propose the use of the available data on the immune ITM cattle coupled with genomic targets from *T. parva* and transcriptomic analyses of the mammalian host response as a combined strategy to reveal novel antigens and protective biomarkers that can be used to develop improved vaccines against ECF [65–67]. Such vaccines will likely be multicomponent formulations, targeting both sporozoite and schizont stages of *T. parva* and incorporating state-of-the-art adjuvants to drive elicitation of specific protective immune responses. Also, success of this strategy might require the evaluation of alternative antigen delivery systems capable of activating bovine MHC class I–restricted cells, and subsequent induction of CD8+ cytotoxic responses associated with protection against ECF.

T. orientalis, also historically known as *T. sergenti* and *T. buffeli*, is considered an emerging parasitic pathogen that poses a severe threat to the cattle industry in Japan, Australia, and New Zealand, among other countries [68]. Anemia is the major clinical sign associated with *T. orientalis* infection, which is detected by pale mucosae, pyrexia, and elevated heart and respiratory rates. These signs are typically associated with a history of cattle being moved into an endemic area for *T. orientalis*. The Chitose and Ikeda types of the parasite are associated with marked anemia, whereas the Buffeli type seems to be less virulent [69]. Interestingly, considering that anemia is the most pronounced sign of oriental theileriosis, it makes this disease more similar to bovine babesiosis than to ECF or tropical theileriosis, and this fact needs to be taken in consideration for clinical differential diagnosis. The recent identification of the *T. orientalis* Ikeda genotype in animals showing anemia in Virginia, US, combined with the presence of competent *Haemaphysalis longicornis* vector ticks in the region, has brought major concerns to the country's cattle industry [70, 71]. Over the years, attempts have been made in Japan and Australia to treat bovine theileriosis with several compounds, such as imidocarb, buparvaquone, and pamaquine, among others. However, the use of these drugs is unpractical for large herds considering their questionable efficacy in field conditions, toxicity, and high costs [72, 73]. Altogether, variation in pathogenicity of parasite genotypes, widespread distribution of competent tick vectors, and impracticality of available treatments pose a real challenge for the development of efficient strategies to control the disease.

No commercial vaccines are currently available to control *T. orientalis*. Early attempts were made to produce a live vaccine using piroplasm parasites; however, this strategy was discontinued due to low efficacy of the approach and concerns on the potential risk of transmitting blood pathogens during vaccination. Therefore, efforts have been concentrated on the identification of novel antigens for subunit vaccines against *T. orientalis*. In that context, partial protection and reduction of clinical signs of acute disease were demonstrated by using subunit vaccines generated from full-length or immunogenic segments of the *T. orientalis* major piroplasm surface protein (MPSP) [74]. However, MPSP is highly diverse, which may affect its wide use as a vaccine antigen. In addition, considering the MPSP diversity, this target has been used to classify *T. orientalis* into several distinct genotypes worldwide [19, 75, 76]. Recent studies on the *T. orientalis* draft genome have revealed interesting features among the Ikeda, Chitose, and Buffeli genotypes that can be explored for vaccine development. As a result, newly identified proteins predicted to be expressed on the surface of *T. orientalis* piroplasms can potentially be evaluated as vaccine antigens [77]. Collectively, recurrent outbreaks of oriental bovine theileriosis in Japan, Australia, and New Zealand in last decades have demonstrated the complexity of this disease [19]. In general, considering that no vaccines are available to control the disease,

outbreaks of *T. orientalis* are generally managed by the use of acaricides to decrease the population of competent tick vectors in a target, affected region. However, this is a risky strategy considering that ticks can develop resistance to acaricides and the use of such drugs can have environmental implications. More studies on the interactions among *T. orientalis*, tick vectors, and cattle are needed to develop sustainable control measures to oriental theileriosis. The ultimate goal is to develop a vaccine, probably containing a cocktail of antigens, to induce a protective immune response in cattle.

In conclusion, the main current strategy to decrease the devastating effects of acute infection with *T. annulata*, *T. parva*, and *T. orientalis* is the use of acaricides to control their respective tick vectors and, to a lesser extent, anti-*Theileria* drugs. Despite the availability of ITM and cell line vaccines to control ECF and tropical theileriosis, respectively, these are far from optimal strategies. Considering the similarities among these *Theileria* parasites, we propose the development of a systemic and multifactorial approach to discover immune mechanisms associated with protection and novel parasite antigens for vaccines. We predict that by focusing on the host aspects of protection, such as diversity of MHC haplotypes and additional genetic factors of the immune response, disappointing results in field trials of antigens identified by T-cell approaches, especially for *T. parva* and *T. annulata*, might be reevaluated. Also, special attention should focus on mechanisms involved in sexual reproduction of these parasites inside the tick midgut that could potentially be explored for the development of transmission-blocking vaccines. In addition, detailed investigation of host-parasite interactions (i.e., protective immune responses and parasite antigens that are targets of such immune responses) in immune animals that survived acute infection, especially for ECF and tropical theileriosis, can potentially provide additional insights for the discovery of novel vaccine antigens and biomarkers of protection. Evaluation of innovative and practical alternative vaccine delivery systems, such as liposomes, virus-like particles (VLP), immune-stimulating complexes (ISCOMS), and nanoparticles, is also crucial for the development of efficient control strategies against tropical theileriosis, ECF, and oriental theileriosis.

Vaccines against bovine babesiosis

Bovine babesiosis is a tick-borne disease caused mainly by the apicomplexan parasites *Babesia bovis*, *B. bigemina*, and *B. divergens*. While *B. bovis* and *B. bigemina* may have worldwide impact, *B. divergens* is found mainly in Europe. Other parasites that can cause bovine babesiosis include *B. major* and *B. ovata*. In contrast to *Theileria* parasites, *Babesia* spp. only invade erythrocytes in their vertebrate hosts, where they reproduce asexually. The definitive hosts of *Babesia* parasites are Ixodid ticks, mainly *Rhipicephalus* spp. for *B. bovis* and *B. bigemina* (such as *R. microplus*) and *Ixodes ricinus* for *B. divergens*. *Babesia* undergoes sexual reproduction in the midgut of the tick hosts and can be transmitted transovarially after invasion of the eggs in the female ticks by kinete stages of the parasites circulating in the hemolymph, a defining feature of *sensu stricto Babesia* spp. [9].

Bovine babesiosis is characterized by fever, anemia, and hemoglobinuria. However, the clinical signs are variable depending on the *Babesia* spp. involved and other factors, such as the age and general condition of the animals. Massive hemolysis and hematuria are typical signs of *B. bigemina* infection. In addition to anemia, *B. bovis* infection causes sequestration of infected erythrocytes in the microvasculature of the host, including those of the brain and lungs, leading to cerebral babesiosis and respiratory distress syndrome, respectively [78, 79]. During acute infection in naïve animals, parasites expand essentially unchecked by the adaptive immune system, which may lead to death when adult cattle are infected. However, young animals are more resistant to acute infections than adult cattle (> 1 year old). The innate immune system of younger animals plays an important role in controlling the disease, especially through clearance of infected erythrocytes in the spleen [80]. Interestingly, IL-12 and IFNγ are released earlier in younger *B. bovis*–infected animals. In addition, they are also more efficient at producing high levels of inducible nitric oxide synthase (iNOS), an effector that is essential for the destruction of the parasite. In fact, the activity of the innate immune system is critical for the survival of infected animals, and it needs to be considered for the development of novel control measures. By contrast, older cattle infected with *Babesia* usually quickly succumb to acute disease, in general about 10–12 days after the onset of infection. However, animals that survive acute babesiosis develop a strong protective adaptive immunity, although they cannot fully eliminate the parasite and become persistently infected [81]. Herds in endemic areas develop a condition known as endemic stability, where few clinical cases are evident, despite high levels of *Babesia* infection in the population (>75%). The occurrence of endemic stability is facilitated by the increased resistance of young calves to infection, combined with maternal antibodies in the colostrum in lactating calves. However, naïve animals, especially adult individuals, cannot be incorporated to these herds in regions with endemic stability without a high risk of succumbing to acute disease [82]. These scenarios require the application of distinct strategies, such as vaccination, tick control using acaricides, and chemotherapeutic treatments in endemic areas. Because of this and other limitations, including the effect of climate change, endemic stability approaches are not considered effective for the long-term control of bovine babesiosis [83, 84]. A tick control strategy based on acaricides is also a frequent approach, especially when eradication of ticks and *Babesia* is attempted, but it is also problematic because of the emergence of drug-resistant ticks and the negative environmental impact of current available acaricide compounds. Antitick vaccines are, however, an interesting alternative for the control of ticks

and bovine babesiosis. A tick vaccine based on the concealed Bm86 antigen showed promising results initially, but there were important drawbacks when applied to control ticks in some countries and their production was essentially discontinued [85]. More effective alternative tick vaccines based on other tick antigens and antigen cocktails are currently under development [86].

Babesia live vaccines based on attenuated parasites are also available for the prevention of *B. bigemina* and *B. bovis* infections, but not for *B. divergens*. These live vaccine strategies evolved from the common practice of "premunition," consisting of the inoculation of blood of an infected animal into naïve recipients, a procedure that resulted in the cotransmission of other blood-circulating pathogens. *B. bovis*–attenuated parasites used in live vaccines are usually prepared by serial rapid passages of a virulent strain in splenectomized calves, and normally 22–25 passages are required. By contrast, the *B. bigemina*–attenuated strains are generated by "slow" similar passages but using spleen-intact calves [87]. The attenuated strains obtained by these procedures are usually poorly virulent for young calves, but they still can cause severe acute babesiosis and high mortality rates when applied to adult animals (> 1 year old). However, vaccinated animals remain persistently infected with the vaccine strains and develop a solid humoral and cell immunity that usually protects them against homologous and heterologous strain challenge [17].

The correlates of protection using live vaccines remain unclear, but several lines of research suggest that protective immunity requires the production of a Th-1 type of response with production of IFNγ, IL-12, and antibodies of the IgG2a subtype [88]. The mechanisms involved in the process of attenuation by serial passages also remain unknown, but it is possible that it involves the selection of preexisting attenuated parasite populations present in the virulent isolates [89]. *Babesia* parasites can also be attenuated by exposure to low doses of radiation, and this is the source of parasites in the vaccines used in Mexico [90]. Once an attenuated *Babesia* strain is obtained, it can be amplified for vaccine production using different approaches. Thus, the *Babesia* vaccines currently available in Australia and South Africa are produced by expansion of the attenuated strain in highly controlled, pathogen-free calves. Live vaccines are also produced by expanding the parasites in *in vitro* cultures, in some countries such as Argentina and Israel [17].

While live *Babesia* vaccines are quite effective to prevent the devastating effects of the acute disease, they present several drawbacks. These limitations include the need to maintain the vaccines in a cold chain, which may be difficult in some endemic areas located in tropical and semitropical regions of the globe, the risk of coinfection with contaminating coinfecting agents, and the possibility for parasite reversion to virulence. In addition, the decision of using attenuated vaccines implies the maintenance of *Babesia* parasites in the herds, which may become problematic if vaccines are based on tick-transmissible parasites. Currently, it is difficult, if not impossible, to differentiate vaccinated from naturally infected animals using serology, and it might be desirable to incorporate markers into the vaccine parasite strain, or to develop a differentiating infected from vaccinated animals (DIVA) vaccine. Taken all these aspects together, future options consist of improving live vaccines by using nontransmissible strains, DIVA markers, or genetically manipulated strains unable to revert to virulence [18].

Alternative options to live vaccines include developing subunit or inactivated vaccines. Evidence for the feasibility of subunit vaccines was collected initially by testing inactivated vaccines based on protein fractions obtained from merozoites or infected erythrocytes, or secreted parasite antigens (SPA) derived from *in vitro* culture of *Babesia* parasites, successfully employed to develop inactivated vaccines against *B. canis* [91]. These vaccines, based on a mix of antigens that were "secreted" by cultured *B. bovis* parasites, showed promising results, but overall, they appeared to be more effective against homologous parasite challenge [92]. Although vaccines based on such antigens are not currently available, they provide a strong rationale for using recombinant versions of antigens in vaccine formulations. As described below in more detail, none of the recombinant antigens so far tested, either alone or in association, proved effective for subunit vaccines against *B. bovis* or *B. bigemina*. However, effective experimental vaccines using recombinant forms of antigens identified in culture supernatants have been developed for *B. divergens* and *B. canis* [93, 94].

Among the abundant vaccine candidates for *B. bovis*, only a few have so far been tested in vaccination-challenge experiments [17]. These include, among a few others, the rhoptry-associated protein-1 (RAP-1), two members of the variable major surface antigens (VMSA), the merozoite surface antigen-1 (MSA-1), and MSA-2c, the 12D3 antigen, and the heat-shock protein 20, either as whole recombinant proteins or selected B- and/or T-cell epitopes from them. Unfortunately, none of the formulations based on these antigens were able to elicit effective and strong protective immunity comparable to live vaccines in animal trials [48, 95–97]. The perceived collective experience indicates that the issues involved in vaccine development against bovine *Babesia* spp. are complex and more research is needed. The lessons so far learned suggest that more knowledge is required on establishing the mechanisms of disease and the correlates of protection in animals resistant to *Babesia* infection, either due to natural processes or due to vaccination with live vaccines. The identification of correlates of protection could help develop screens to identify protective antigens. Combined, the identification of the correlates of protection and protective antigens may lead to the rational design of efficient subunit vaccines.

While protection against acute babesiosis may be difficult to achieve, novel alternative vaccine approaches have also been emerging [98, 99]. This includes the development of vaccines that can block transmission of the parasites. Several transmission-blocking vaccine (TBV) candidates

have been identified and characterized recently in *B. bovis* and *B. bigemina*, including members of the CCp and 6Cys families, and HAP2 [100–104]. Most of these antigens are also conserved in related parasites such as *Plasmodium* spp. and *Theileria* spp. [39, 101, 105, 106] and have been shown to be effective in reducing transmission upon vaccination in different models [107]. Antigens differentially expressed in kinete stages are also possible candidates for developing TBV. These include the *B. bovis* protein BboKSP, encoded by the gene identified as BBOV_I002220 [108], and its *B. bigemina* homologous, BbiKSP, encoded by the BBBOND_0206730 gene [102]. Such TBV candidates are exclusively expressed in tick stages and this unique expression profile could be the result of an evolutionary adaptation to escape the vertebrate host immune responses, in addition to adapting for functional requirements. Furthermore, vaccination with such concealed antigens that have not been exposed to the pressure of the bovine host immune system may be restricted in their ability to vary antigenically. This is clearly an example of confronting the parasite with a challenge that it did not face during its long coevolutionary history between the arthropod and vertebrate hosts. An advantage of this approach is that these antigens are widely conserved and not exposed to the selection pressure of the vertebrate host immune system, since they are only expressed in tick stages, which makes these parasite targets reasonable for intervention to block transmission. However, it is likely that frequent revaccinations will be required in order to maintain high antibody titers against such tick-stage–specific antigens, since natural boosts will not occur due to their differential expression in tick-stages. A realistic approach, based on the current state of the art, would be to generate a vaccine containing blood-stage antigens and tick-stage antigens in which blood-stage antigens elicit an immune response that alleviates the devastating effects of acute babesiosis and allows time for the animals to develop a strong adaptive immune response, and tick-stage antigens to prevent parasite transmission between hosts. This combination of recombinant vaccine including both blood-stage and tick-stage antigens could be the most promising approach to control bovine babesiosis in the future. Combining a blood-stage vaccine capable of controlling clinical disease with a TBV would improve animal production and keep the parasite numbers low in the vaccinated areas.

Vaccines against bovine coccidiosis caused by Eimeria

The genus *Eimeria* comprises about 200 species that infect a large variety of vertebrates around the world. While these parasites are strictly host specific and their distribution follows that of their hosts, one host can be infected by several species at the same time. *Eimeria* has a monoxenic life cycle with endogenous and exogenous developmental stages. The endogenous phase generally takes place at species-dependent predilection sites of the digestive tract, where intracellular asexual and sexual multiplication occurs. The latter results in the generation of a zygote around which a resistant protective oocyst wall develops. When oocysts are mature, they rupture their host cells, are released to the intestine lumen, and shed with the feces. Shed oocysts need to sporulate outside the host to become infective, which takes one to four days under ideal temperature, moisture, and oxygen levels, or up to several weeks, under less favorable conditions. Upon ingestion of sporulated oocysts by the host, parasites excyst in the intestine and invade mucosal intestinal cells, where new cycles of multiplication begin [109].

Oocysts are very resistant and last for months in the environment. They are also highly infective, and a few oocysts can establish an infection in a susceptible host that will yield millions of oocysts in the next life cycle. These features make *Eimeria* spp. extremely successful parasites, especially in areas where animals congregate or are crowded, and feces are more concentrated on the ground [110].

Thirteen *Eimeria* species are known to infect cattle, three of which, *E. zuernii*, *E. bovis*, and *E. alabamensis*, are pathogenic, and have also been detected in water buffaloes [111]. Clinical signs, mainly diarrhea and anorexia, are present in young calves, between 6 and 12 months old, while adult cattle are normally asymptomatic, unless immunosuppressed due to stress or other diseases. Depending on the level of exposure, general condition, genetic susceptibility, and other factors, clinical signs can vary from self-limiting, in which animals recover without treatment, to fast deterioration and death. Heavy infections of calves with *E. zuernii* can lead to nervous coccidiosis with tremors, convulsions, and other central nervous system signs, resulting in high mortality rates [110].

Production losses are associated with increased calf mortality, as well as delayed fertility caused by a retarded weight gain and lower final weight [112]. Prevention is considered preferable than treatment from economic and animal welfare points of view and is carried out avoiding overcrowding, improving nutrition, and increasing hygiene of feeding and watering equipment [110]. Additionally, anticoccidial drugs that inhibit parasite growth have proved useful not only to treat clinical disease but also as metaphylactic treatment to improve productive parameters in herds naturally exposed to *Eimeria* spp., as well as to decrease environmental contamination with oocysts [113, 114].

Bovine coccidiosis is an extremely underresearched parasitic disease, reflected by the scarcity of published reports on vaccine development. The available evidence supports the potential of the approach of using *Eimeria* sp. oocysts as immunogen in vaccine formulations against bovine coccidiosis. Thus, inoculation of calves with *E. alabamensis* oocysts induced protection upon field exposure to the parasite [115]. Also, a vaccine based on sonicated formalin-inactivated *E. bovis* sporulated oocysts elicited high levels of specific antibodies and protection upon challenge in calves [116]. Finally, UV inactivation of *Eimeria* sp. oocysts was applied to produce an experimental protective immunogen against coccidiosis in lambs [117].

However, further exploration of this and related lines of research have remained essentially neglected. Conversely, successful vaccines against poultry coccidiosis have been used for 70 years, based on oocysts of wildtype and, more recently, attenuated avian *Eimeria* strains [118]. Also, important research efforts devoted to whole-genome sequencing, transcriptomic analysis, characterization of vaccine candidate antigens for subunit vaccines, and gene editing have been so far exclusively concentrated on avian *Eimeria* species [119, 120]. Importantly, as in the case of avian coccidiosis vaccines, a desirable vaccine against bovine coccidiosis should be based on parasite antigens that are accessible to the host intestinal immune system and that elicit an immune response that can decrease parasite proliferation, as well as a strong memory response to protect animals from reinfection [121]. Considering the current research status, a vaccine against bovine coccidiosis does not seem to be an achievable goal in the near future, but this area of research will eventually benefit from the far more rapid advances in avian coccidiosis vaccines.

Vaccines against bovine toxoplasmosis and neosporosis

Toxoplasmosis is a very common zoonotic disease caused by *Toxoplasma gondii*. This coccidian is an obligate intracellular parasite that is very apt to infect nearly all warm-blooded animals and can invade many different types of cells. In fact, it was estimated that 92% of the world cattle population harbors antibodies against *T. gondii* [122]. As it is such an important global zoonosis, it was the target of "one health approaches" for vaccine development [123]. Although toxoplasmosis is not generally considered a high-risk disease for immunocompetent human populations, it can cause abortions in pregnant women and acute disease in immunocompromised individuals. In addition, it was found that the parasite may alter brain chemistry by affecting the production of dopamine [124]. The usual treatment for toxoplasmosis in humans consists of a combination of pyrimethamine with sulfadiazine. Felines are the key and definitive hosts for *T. gondii*. Cats can acquire the parasite by predating on rodents, birds, and other species and are the only definitive host, where the sexual life cycle of the parasite can occur in their guts, resulting in the shedding of *T. gondii* oocysts in feces. Transmission of *T. gondii* may occur through consumption of sporulated parasites derived from oocysts deposited by cats in contaminated food, such as vegetables or water [125], or raw or undercooked meats containing *T. gondii* tissue cysts [126].

Toxoplasmosis is also considered a mild disease in bovines, which are usually able to mount strong immunity against the parasite, and the development of vaccines for this species is not considered a priority [127, 128]. However, whether infection of cattle with *T. gondii* constitutes a relevant risk factor to humans remains to be established. While toxoplasmosis is not usually regarded as a risky disease for cattle, it is known to be an important cause of abortion in sheep, and a live vaccine for this species is available [129]. In addition, swine meat contaminated with *T. gondii* is also an important human health risk. Therefore, addressing *T. gondii* control in pig herds may be of relevance [130, 131].

A one health approach for toxoplasmosis is based on the development of livestock vaccines that reduce infection of the cat and therefore shedding into the environment, which, in turn, can help reduce the burden of this potentially dangerous disease in immunocompromised patients and pregnant women. Also, the strategic application of vaccination of food animals was suggested as a means of preventing/reducing viable tissue cysts in meat, making it safer for human and animal consumption [132]. Numerous *T. gondii* vaccine candidate antigens have been identified, and an attenuated genetically manipulated KO strain was also tested, as recently reviewed in [123]. An important factor for developing a *T. gondii* vaccine is selecting the most appropriate life stage of this parasite as an effective target. Despite intensive research, no subunit or killed parasite vaccines are currently available, but studies on protective immune mechanisms suggest that a successful subunit or inactivated vaccine candidate should be able to stimulate key protective immune responses involving the production of IFNγ and activated CD4+ T cells and then activated CD8+ T cells, followed by the detection of specific antibodies [133]. In addition, interventions aimed at interfering production and/or sporulation of oocysts may help limit spreading of *T. gondii* in the environment, resulting in turn in a reduction of the incidence of toxoplasmosis in humans and livestock. Recent work identified genes encoding for proteins involved in the formation of sexual stages and oocyte walls that can be candidates for transmission-blocking vaccines against *T. gondii*. Furthermore, it was demonstrated that a genetically engineered nontransmissible mutant *T. gondii* strain lacking expression of the HAP2 sexual stage gene was able to generate immune responses that prevent transmission of *T. gondii* by cats (the only known definitive host of the parasite), thus demonstrating the feasibility of developing TBVs against *T. gondii* [134].

The causative agent of bovine neosporosis is *Neospora caninum*, an apicomplexan protozoon belonging to the Sarcocystidae family, which is highly related to *T. gondii*. This parasite was first described in the 1980s as an agent of neuromuscular disease in dogs and only later recognized as a major agent of abortion and neonatal mortality of cattle worldwide. Cattle producers experience annual losses of millions of dollars due to *N. caninum*–associated abortions in dairy and beef cows and other indirect costs [135, 136].

As other members of the Sarcocystidae family, *N. caninum* displays a two-host, predator-prey life cycle. The sexual stage is completed in the digestive track of dogs and other canids that excrete unsporulated oocysts with their feces. Under adequate environmental conditions, oocysts undergo sporulation and become infective. Ingestion of

oocyst-contaminated water or food leads to infection of a large variety of intermediary hosts, including cattle, sheep, deer, bison, water buffalo, and even wolves and dogs. Tachyzoites formed in the intermediary host gut disseminate by moving between tissues or through the bloodstream, invade different tissues, and reproduce intracellularly, inside a parasitophorous vacuole. Eventually, responding to a host signal, they undergo stage conversion into bradyzoites, which form a dormant thick-walled tissue cyst stage and slowly divide, until reaching approximately 100 parasites per cyst. The parasite life cycle is completed when a canid ingests meat infected with tissue cysts. Most importantly, *N. caninum* tachyzoites are efficiently transmitted through the placenta in several intermediary hosts and, indeed, vertical infection is the main transmission route in cattle. An infected dam produces 90% infected fetuses. Depending on factors such as the time of infection and their immunological status, infected fetuses can die in utero or can be resorbed, mummified, autolyzed, still borne, or born alive. Infected born calves show occasionally neurological signs, weakness, and/or underweight but are generally asymptomatic. They remain chronical carriers and females are able to transmit vertically the parasite to the next generation. Transplacental transmission can take place upon a primary infection of a pregnant dam after oocyst ingestion, but more commonly, upon recrudescence of a preexistent infection during pregnancy. Abortions can be epidemic or endemic and are caused by parasite-provoked placental necrosis that hampers fetal survival, primary tissue damage due to parasite multiplication in the fetus and/or placental inflammation, and release of maternal proinflammatory cytokines that provoke maternal expulsion of the fetus [4, 137].

There are no commercially available drugs to protect against bovine neosporosis, albeit a considerable number of compounds have been tested in animal models or in cattle under controlled conditions with some promising results. Drug therapies would be useful to prevent abortions due to primary infections or recrudescence of preexistent ones and to avoid the establishment of chronic infections in calves born to infected dams [138]. However, the need for prolonged preventive treatments would make drug therapies uneconomical, with the additional risk of generating unacceptable residues in meat and milk. Currently recommended control measures include selective culling of infected cattle, embryo transfer, limitation of the access of canids and their feces to water and food destined for cattle consumption, and preventing canids from eating tissues resulting from abortions. However, these measures are either highly costly or only partially effective due to the existence of a sylvatic cycle of the parasite in birds, rodents, and other mammals. Vaccination to limit vertical transmission and abortion is considered the most cost-effective measure to control neosporosis [139].

A vaccine (Bovilis Neoguard) based on killed tachyzoites was commercialized for a few years and shown to induce anti–*N. caninum* antibodies, be safe, and not to affect meat quality in feedlot steers [140]. However, it only yielded partial reduction in abortion rates and has been removed from the market [141, 142].

As in the case of sheep and human toxoplasmosis, natural exposure to the parasite before pregnancy is the most efficient way to prevent abortions in cattle; hence, a live vaccine appears as a simple and attractive tool for the control of bovine neosporosis [143]. Indeed, vaccination of pregnant dams with an attenuated strain, isolated from an asymptomatic *N. caninum*–infected calf from Australia, prevented abortion upon challenge with a virulent parasite strain. This effect was not observed in cows vaccinated with a lysate of the attenuated strain. Interestingly, while both live and lysate preparations elicited a strong antibody response, only live vaccination induced proliferation of CD4+ T cells and production of IFNγ, suggesting that a cellular response is needed for protection [144, 145]. The production of live attenuated vaccines can benefit from the availability of *in vitro* culture protocols for *N. caninum* and is considered to have a high chance of success; however, such vaccines have not yet reached the market [143].

Importantly, some disadvantages of live vaccines, such as maintenance of the parasite in immunized herds with potential for pathogenicity reversion, costly production and distribution, and short storage life, render a subunit vaccine an attractive alternative. An important amount of research has been carried out to identify *N. caninum* proteins and parasite fractions with a role in host cell invasion and/or differentiation from the tachyzoite to the bradyzoite stage and to develop vaccine formulations that generate humoral and cellular immunity against these components. The majority of vaccination/challenge experiments have been carried out in a mouse model, either using nonpregnant mice to evaluate acute disease and infection of the central nervous system or pregnant mice to assess transplacental transmission and female/offspring health. However, immunological differences between mice and cattle restrict the validity of the results to preliminary indications of potential protective effects. On the other hand, while experiments in cattle are constrained by their high costs, sheep have emerged as a cost-efficient small ruminant model for neosporosis, since they can also experience abortions due to *N. caninum* infections. So far, only live attenuated vaccines have conferred convincing protection; thus, further efforts are needed to produce an efficient subunit vaccine [139, 146, 147].

Unraveling new targets for vaccine development using molecular biology tools is not as advanced in *N. caninum* as in other more extensively studied apicomplexan parasites. Genomic sequencing of geographic isolates has thrown light on interesting aspects of the parasite population structure [148]. Conversely, the application of other advanced methodologies, such as "omics" approaches to antigen discovery, has been hampered by the incomplete annotation of the *N. caninum* genome, where a large number of functionally significant proteins were annotated as "hypothetical" and have thus remained unnoticed [149].

Bioinformatics tools have shown that some of these previously uncharacterized proteins are likely involved in essential biological processes such as parasite motility, adhesion, invasion, signaling, and interaction with host cells and thus constitute attractive vaccine and drug targets [150].

Validation of the functional relevance of these and other antigens could be carried out by knock-out experiments using available gene-editing tools, including stable transfection and CRISPR/Cas9 technology [151, 152]. Gene editing could also be applied to produce attenuated vaccine strains in which genes encoding major virulence factors have been deleted, and/or with introduced markers to be used as DIVA vaccines.

Progress in the advancement of bovine neosporosis control strategies has been hampered by the status of *N. caninum* as a nonmodel organism. This disease will continue to pose a burden for cattle producers around the world and generate substantial reproductive and productive losses until consistent financial support is granted for the production of safe and efficacious vaccines that limit *N. caninum* proliferation and dissemination.

Vaccines against besnoitiosis and sarcosporidiosis

Bovine besnoitiosis (elephant skin disease) is an emerging acute and chronic disease caused by the apicomplexan intracellular protozoan *Besnoitia besnoiti*, a cystogenic coccidium. Illness begins with fever, followed by warm, painful swellings ventrally (anasarca). *B. besnoiti* merozoites invade and proliferate in macrophages, endothelial cells, and fibroblasts producing characteristic large, thick-walled cysts filled with bradyzoites, causing vasculitis and thrombosis. These stages, together with the oocyte and the tachyzoite, are the infectious parasite forms. The subsequent cellular destruction and immune response lead to the characteristic acute signs of anorexia, lethargy, generalized skin edema, and the chronic signs of alopecia and scleroderma. Other signs, such as swollen lymph nodes, diarrhea, photophobia, rhinitis, and orchitis, among others, can also occur. The parasite affects cattle production in several European, Asian, and African countries. Although mortality is considered low (less than 10% of the cases), convalescence is slow in severe cases. Therefore, this parasite may be responsible for important economic losses for the cattle industry [153].

Cattle are known to be intermediate hosts; however, definitive hosts and mode of transmission remain unknown, and there are substantial knowledge gaps on the life cycle and biology of this parasite. Although the route of transmission remains unclear, cattle are usually isolated and protected from biting insects and ticks and then treated symptomatically. Chemotherapy remains very limited and is only minimally effective. Both antimony and sulfanilamide complexes prevented cyst development by *B. besnoiti* in rabbits, and oxytetracycline may have some therapeutic value if given early in the disease course [154]. However, there is still no effective drug available for the control of besnoitiosis in cattle.

South Africa and Israel have used a live attenuated vaccine against bovine besnoitiosis [155]. An extensive field trial that was carried out in South African farms, where the disease was severe, found that 100% of the cattle inoculated with a blue wildebeest strain of *B. besnoiti* vaccine were protected from the clinical form of the disease over an observation period of 1–4 years [156]. In Europe, at present, only reliable diagnosis together with herd-management measures are available to avoid that noninfected herds acquire the infection due to trade with infected animals [132]. These data suggest that developing a safer live or subunit vaccine is an achievable goal. However, the nature of protective immune responses remains unknown and the identification of novel targets for chemotherapeutic or immunological interventions remains severely limited by the lack of genomic and transcriptomic data [157]. Bradyzoite-specific antigens that could be candidates for vaccine development were also identified, but little progress has been documented on the development of subunit vaccines against this parasite [158]. Altogether, this indicates that additional research is needed in order to generate effective subunit or killed vaccines that can ameliorate the impact of this parasite in bovine populations at risk.

Sarcosporidiosis is another disease in livestock with zoonotic potential. The disease, caused by apicomplexan parasites of the genus *Sarcocystis,* is characterized by the formation of sarcocysts containing infective bradyzoites that range in size from micrometers to a few centimeters, in the muscle or nervous tissue of their hosts. *Sarcocystis* spp. infections are quite prevalent in farm animals; however, there have been few reported outbreaks of clinical disease. Most animals are asymptomatic, and the parasite is discovered only upon slaughter.

The genus *Sarcocystis* contains more than 100 species that may differ in host specificity and pathogenicity but have a typical coccidian life cycle involving merogony, gametogony, and sporogony. The identification of different species is achieved by molecular studies and cyst wall morphology. *Sarcocystis* spp. normally develop in obligatory two-host cycles consisting of an intermediate host (prey) and the final host (predator). Usually, the herbivore is the intermediary host, while a carnivore (i.e., dog, cat, human, etc.) is the definitive one. Species-specific prey-predator life cycles have been demonstrated for cattle-dog (*S. cruzi*), cattle-cat (*S. hirsuta*), cattle-human (*S. hominis*), pig-human (*S. suihominis*), and others. Human ingestion of sarcocysts of *S. suihominis* or *S. hominis* in uncooked pork or beef, respectively, may cause nausea, abdominal pain, loss of appetite, vomiting, and diarrhea lasting as long as 48 hours [4, 159].

As stated above, *Sarcocystis* spp. infections are generally considered highly prevalent worldwide, but of low pathogenicity. However, induced infection with *S. cruzi* sporocysts from canine feces may cause acute disease in

calves, microscopic cysts in myocardium, as well as eosinophilic myositis in cattle, and abortions, stillbirths, and deaths in pregnant cows. Similar pathogenicity has been demonstrated for *S. tenella* in lambs and ewes and for *S. miescheriana* in pigs. The manifestation of clinical disease may depend on the immune status of the host and the dose of sporocysts. A procedure involving preventive immunization using small doses of sporocysts appears to prevent the development or reduce severity of clinical disease in sheep when challenged later with large doses (premunitive immunity). Pigs can also have persistent acquired immunity after immunization infections. Importantly, cattle and llama meat containing visible cysts are usually condemned upon inspection after slaughter, with consequent economic losses [160].

No vaccines to control *Sarcocystis* spp. infections are currently available for any livestock species, and little research has been performed in the identification of vaccine candidate antigens. Importantly, methods to initiate cultures from sporocysts and merozoites and for cryopreservation of various *Sarcocystis* spp. are now emerging [161]. This will facilitate our understanding of the antigenic composition of the parasites and the identification of novel vaccine candidates. A study by Howe *et al.* [162] demonstrated the expression of surface antigens of the SAG family that could constitute attractive vaccine candidates in *S. neurona*, a species that infects and causes encephalitis in horses. SAG antigen genes were also recently shown to be transcribed in bradyzoites of *S. aucheniae*, a *Sarcocystis* species that produces macroscopic cysts in South American camelids [160]. However, it should be assessed whether vaccines are needed, for which species, and which life stages of the parasite should be targeted.

Livestock become infected by sporocysts from the feces of carnivores. The main strategy to control sarcocystosis is based on interrupting the parasite life cycle. Thus, simple preventive measure can be taken to control this disease. For instance, dogs and other carnivores should be precluded from eating raw meat, offal, or dead animals. There is no specific therapeutic method for sarcocystosis, but anticoccidial drugs are an option for prophylactic or curative chemotherapy, though the efficiency of drugs on different life stages may vary [163]. Noteworthy, it has been shown that infected pork and beef could be made safe for consumption by cooking at 70°C or freezing. In summary, *Sarcocystis* spp. can be considered as neglected understudied parasites. Many research gaps remain, and there is a scarcity of options for the prevention and treatment of this disease.

Vaccines against bovine cryptosporidiosis

Bovine cryptosporidiosis is caused by infection of preweaned calves with *Cryptosporidium parvum,* leading to gastroenteritis and profuse diarrhea. The disease is occasionally fatal and animals surviving the infection do not entirely compensate growth retardation, resulting in considerable production losses. The parasite is ubiquitous in all cattle farms but is of particular importance in intensive management systems, such as dairy farms. In addition to *C. parvum*, the species *C. bovis* and *C. ryanae* may be detected in weaned calves but have not been found associated with clinical disease [164]. A fourth species, *C. andersoni*, is occasionally detected in the abomasum of adult cattle and considered to be asymptomatic, though reduced weight gain and milk production associated with this infection have been reported [165]. In neonatal sheep and especially goats, cryptosporidiosis can lead to high morbidity (100%) and mortality (50%), and the species most frequently detected is *C. parvum*. In pigs, infection with *C. parvum* leads to diarrhea of 3–5 days duration and is most frequently observed in piglets or starter pigs [10, 164].

Cryptosporidium displays a monoxenous life cycle. The sporulated oocyst stage is excreted with the feces into the environment where it remains infective for a prolonged period and is resistant against a large variety of environmental conditions. Transmission of oocysts occurs via the fecal-oral route either directly through contact with feces of infected hosts or indirectly by uptake of contaminated water or food. After ingestion of oocysts, excystation takes place and results in the release of four sporozoites that infect endothelial cells of the gastrointestinal tract. There they develop into type I meronts, where asexual propagation into eight merozoites takes place. Merozoites either autoinfect neighboring endothelial cells spreading the infection to other sites of the intestine or develop into type II meronts starting sexual reproduction. The latter finally results in the production of thin-wall and thick-wall oocysts. Thin-wall oocysts release sporozoites that autoinfect intestine cells, further promoting parasite multiplication in the host individual, whereas thick-wall oocysts are excreted and infect other host individuals, promoting dissemination of the parasite in the population [10].

The completion of the life cycle of the parasite is facilitated by three factors. First, multiple consecutive propagation cycles, including two autoinfection loops, result in massive parasite multiplication and an enormous parasite load in the host. This, in turn, ensures efficient oocyst excretion and dissemination into the environment. Thus, it has been reported that a single calf excretes up to about 600 million oocysts per day [166]. Second, oocyst persistence under a wide range of environmental conditions is ensured by a thick wall formed by four layers composed of acidic polysaccharides, glycoproteins, and lipids. In addition, oocysts are resistant to chlorine-based disinfectants commonly used to sanitize drinking water and swimming pools. Third, a very low infective dose of only 17 oocysts secures establishment of infection in calves after parasite ingestion [167]. On the one hand, these characteristics ensure completion of the life cycle and parasite survival, while, on the other hand, they result in massive environmental contamination with oocysts. As *C. parvum* is zoonotic, the study of oocyst viability and environmental

transmission routes is essential for a one health approach to tackle animal and human cryptosporidiosis [123].

Calves can be infected immediately after birth. Oocyst excretion may be observed as early as 2 days after ingestion (prepatent time) and excretion may extend from 1 to 14 days (patent time). A dose dependency has been suggested, as the number of oocysts ingested has been observed to correlate with those excreted. Furthermore, the younger the age of the calf at oocyst intake, the longer the excretion period and severity of disease [168]. Importantly, in artificial rearing, all calves become infected at some point during the preweaning phase. Besides liquid diarrhea, clinical signs include dehydration, loss of appetite, lethargy, and abdominal pain and may be fatal. In severe cases, recovery of calves may take up to three to four weeks during which restoration of intestine absorption of nutrients occurs [169]. Currently, the treatment of cryptosporidiosis of calves is palliative as curative drugs are not available. The only licensed drug is halofuginone, which is used for preventive treatment reducing oocyst excretion and severity and duration of diarrhea [170].

C. parvum is a zoonotic parasite that also causes human cryptosporidiosis, characterized by profuse and prolonged watery diarrhea, abdominal pain, malabsorption, fever, nausea, and vomiting. Besides the zoonotic *C. parvum*, the disease is also caused by the anthroponotic *C. hominis*, which is confined to humans. Recent reports suggest that the majority of water-borne outbreaks, which include recreational and drinking water, are caused by *C. hominis*, whereas food-borne outbreaks are mostly caused by *C. parvum* [171]. The disease in humans is self-limiting and may last up to two weeks in healthy immunocompetent individuals. By contrast, in immunocompromised patients, such as HIV patients, young children, and the elderly, the disease may be difficult to control and often leads to death. In industrialized countries, regular outbreaks are reported and result in considerable economic costs. In developing countries, the largest disease burden of cryptosporidiosis is carried by young children. About 1.2 million children under five years of age worldwide die due to diarrhea [172]. It has been reported that in global outbreaks between 2004 and 2010, in 60% of cases, the responsible etiological agent was *Cryptosporidium* spp. [173].

Research on *Cryptosporidium* lags behind in a number of aspects with regard to other apicomplexan protozoans of veterinary or human importance. This is because only recently methods for *in vitro* cultivation of *C. parvum*, an animal model to reproduce its life cycle, and a genome-editing tool based on the CRISPR/Cas9 system have been established [174, 175]. It can be foreseen that these developments will greatly facilitate a rational approach to vaccine development. Thus, genome editing and the established animal model will allow functional studies of parasite proteins and antigens to assess their suitability as vaccine candidates. Furthermore, the development of stem-cell–derived epithelial organoids has recently been reported in which the parasite propagates and completes its life cycle [176]. The availability of such *in vitro* cultivation systems will allow testing parasite neutralization by antisera raised against vaccine candidates before their use in vaccination trials [177].

For the above-mentioned reason, whole-genome sequencing (WGS) has been likewise hampered, as the relatively low numbers of oocysts usually isolated from feces could, due to lack of an *in vitro* culturing system, not be propagated to numbers suitable for genome sequencing. However, recently, protocols that allow the purification of large numbers of oocysts and the amplification of the recovered genome to quantities suitable for WGS sequencing have been established [178]. This allowed the sequencing of an increasing number of diverse *Cryptosporidium* isolates, revealing exciting novel insights with respect to gene duplications, genetic recombination, population genetics, and the existence of "cryptic species" within *C. parvum* and other *Cryptosporidium* spp. [179].

Currently, no vaccine to protect against cryptosporidiosis in calves or humans is available. A vaccine against bovine cryptosporidiosis would be highly desirable to prevent disease thereby favoring animal health and to decrease dissemination of oocysts into the environment, reducing the risk to public health [123]. A considerable number of potential parasite antigens have been identified by traditional molecular approaches through screening of expression libraries for immunodominant antigens, using sera of infected hosts [180]. As *in vitro* culturing will likely be restricted to some laboratories, a convenient approach for the identification of additional vaccine candidates without parasite cultivation is reverse vaccinology. Indeed, by focusing onto the GPI-anchored proteome, this approach has allowed identification of a number of hitherto unknown vaccine candidates for subsequent use in vaccination trials [181, 182]. Interestingly, an exclusive immunoinformatics approach to design a multisubunit vaccine against *C. parvum* has been recently reported [183].

When conceiving a vaccination strategy that imparts protection to neonatal calves, the following considerations are imperative. Like many farm animals, bovines possess a syndesmochorial placenta, and the fetus does not receive immunoglobulins through transplacental transmission. Instead, passive transfer of immunoglobulins to calves is achieved by feeding of the first milking colostrum. Considering this, a strategy of active immunization of calves is unlikely to be successful. This is so, first, because the immune system of calves is still immature and, second, calves are already exposed to the infection directly after birth leaving insufficient time to mount an immune response that will protect within the first three weeks at which infection typically occurs [184]. Notwithstanding, immunization of calves shortly after birth with gamma-irradiated oocysts has been done. Irradiation prevented development of inoculated oocysts and no clinical signs were observed following immunization. After challenge at day 21, calves were shown to be protected against subsequent oocyst excretion and clinical cryptosporidiosis [185]. However, as calves are

infected directly after birth and predominantly excrete oocysts during their first three weeks of age, the protection generated after this period is unsuitable to prevent calf cryptosporidiosis in the field.

Active immunization of pregnant cows is considered a more promising approach. Immunized cows generate protective antibodies in hyperimmune colostrum, which, when taken up by calves, results in their immediate passive immunization. In two independent studies, immunization of pregnant cows was done using the vaccine candidate p23. After ingestion of hyperimmune colostrum and subsequent challenge of calves, a substantial reduction in the number of excreted oocysts of 98% and 90% was observed [186, 187]. Importantly, no diarrhea or other clinical signs were determined as compared to control groups. Vaccination of pregnant cows with the oocyst surface antigen Cp15/60 resulted in a high titer of specific antibodies in sera and hyperimmune colostrum. After hyperimmune colostrum ingestion, anti-CP15/60 antibodies in sera of calves were found to correlate with the titer of anti-CP15/60 antibodies in colostrum [188]. However, in this study, no subsequent challenge was carried out to test the protection of calves.

Oral application of protective antibodies as a strategy of passive immunization has been studied extensively in a mouse model. It has been shown that passive immunotherapy is effective in reducing the number of intestinal infective oocysts in mice. In this study, experimentally infected neonatal mice were treated with the whole whey or purified specific immunoglobulin isotypes IgG1 and IgA from hyperimmune colostrum of a cow immunized with *C. parvum* oocysts. Oocyst shedding and occurrence of diarrhea were significantly decreased, compared with mice that had received the corresponding immunoglobulin isotypes of control colostrum [189].

Egg yolk antibody IgY can be generated in large quantities and low costs with relative ease and has been extensively used for the treatment and prevention of various infections in animals and humans [190]. Anti-*Cryptosporidium* IgY antibodies may be generated by immunization of chicken, harvested from eggs, and later fed as supplement to newborn calves. This strategy has been successfully applied to viral and bacterial enteric pathogens [191]. In contrast to immunization of pregnant cows, the advantage of this strategy is that it can be applied not only as an immunoprophylaxis to prevent disease but also as an immunotherapeutic measure. In two independent studies, anti-*Cryptosporidium* sporozoite IgY antibodies were orally applied to SCID and C57BL/6 mice, respectively, and in both, a significant reduction of oocyst shedding was observed [192, 193]. However, this vaccination strategy has not yet been tested in cattle.

There is a consensus that a vaccine against bovine cryptosporidiosis is feasible and will bring significant benefits with respect to animal production, animal welfare, and public health. Research on *C. parvum* has made a significant jump in the last few years, and thus, it can be expected that this is an achievable goal in the near future.

Conclusions

Apicomplexan parasites affect cattle globally, posing an important threat for the production of much needed animal-based food resources. It is widely recognized that vaccination approaches are the most effective methods for the control of infectious diseases, including apicomplexan parasites. Some of these parasites are also zoonotic, and a "one health" vaccination approach is required in most cases. Thus, an important remaining goal is diminishing the risk imposed by these parasites to humans by reducing the level of parasite burden/contamination in the herds, the environment, and in the food chain through vaccination.

In general, parasitic diseases caused by apicomplexan parasites have so far remained neglected, and greater awareness about the greater challenges posed by these parasites needs to be promoted through research, education, and outreach activities. This will likely enhance the interest among policy makers and attract the next generation of researchers to work in this area resulting in enhanced capacity to manage these parasitic infections. Furthermore, efforts to increase awareness of these parasites should result in increasing funding for research aimed at finding new and improved prevention and/or control methods. As research efforts into a number of these parasites and parasitic diseases remain in their infancy, adequate and feasible vaccine approaches and strategies should be first identified for each of these parasites. For instance, while infections may require transmission-blocking strategies, others may require prevention of acute disease, or a combination of both approaches.

Regardless of the strategy used, vaccine development essentially requires the identification of antigens that can elicit protective responses and effective methods for delivery. In order to be practical, vaccines also need to be affordable, stable under reasonable environmental conditions, nontoxic to the host, environmentally friendly, and easily available. However, basic knowledge of the biology of apicomplexan parasites and the nature of the protective immune mechanisms is lacking in most cases. Addressing the control of these parasitic diseases requires epidemiological assessments, predictive disease modeling, novel diagnostic systems, and new vaccines and effective drugs. The roadmap for the development of novel vaccines should also include the definition of immune correlates of protection and application of full genome sequencing coupled with novel "omics" approaches. These methods, integrated with genetic analysis using state-of-the-art gene-editing approaches, should result in the identification of novel vaccine candidates. Vaccine delivery regimens and adequate adjuvants also need to be defined for each case, and a pipeline of candidate antigens and their priority for testing should be established. Appropriate vaccine testing models should also be developed to expedite vaccine development and minimize costs.

Altogether, despite the high impact and neglected status of most diseases caused by apicomplexan parasites, the goal of developing effective vaccines against these parasites

remains feasible in the light of recent progress in the fields of vaccinology, immunology, molecular biology, and bioinformatics. Enhanced awareness, improved research funding, higher researcher numbers, and enhanced global collaboration will no doubt lead to the development of effective and sustainable control measures against these important parasitic diseases of cattle in the future.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

CES conceived and drafted the manuscript; MFC, LS, RGB, HFA, and CES contributed sections of the manuscript; MFC, LS, RGB, VR, BMC, and CES provided intellectual input and revised the work.

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