International Journal for Parasitology xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

International Journal for Parasitology

journal homepage: www.elsevier.com/locate/ijpara

Invited Review

Advances in the application of genetic manipulation methods to apicomplexan parasites

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ARTICLE INFO

Article history:
Received 23 July 2017
Received in revised form 24 August 2017
Accepted 24 August 2017
Available online xxxx

Keywords:
Apicomplexan
Genetic manipulation
Transfection
Gene editing
CRISPR/Cas9

ABSTRACT

Apicomplexan parasites such as *Babesia*, *Theileria*, *Eimeria*, *Cryptosporidium* and *Toxoplasma* greatly impact animal health globally, and improved, cost-effective measures to control them are urgently required. These parasites have complex multi-stage life cycles including obligate intracellular stages. Major gaps in our understanding of the biology of these relatively poorly characterised parasites and the diseases they cause severely limit options for designing novel control methods. Here we review potentially important shared aspects of the biology of these parasites, such as cell invasion, host cell modification, and asexual and sexual reproduction, and explore the potential of the application of relatively well-established or newly emerging genetic manipulation methods, such as classical transfection or gene editing, respectively, for closing important gaps in our knowledge of the function of specific genes and proteins, and the biology of these parasites. In addition, genetic manipulation methods impact the development of novel methods of control of the diseases caused by these economically important parasites. Transient and stable transfection methods, in conjunction with whole and deep genome sequencing, were initially instrumental in improving our understanding of the molecular biology of apicomplexan parasites and paved the way for the application of the more recently developed gene editing methods. The increasingly efficient and more recently developed gene editing methods, in particular those based on the CRISPR/Cas9 system and previous conceptually similar techniques, are already contributing to additional gene function discovery using reverse genetics and related approaches. However, gene editing methods are only possible due to the increasing availability of in vitro culture, transfection, and genome sequencing and analysis techniques. We envisage that rapid progress in the development of novel gene editing techniques applied to apicomplexan parasites of veterinary interest will ultimately lead to the development of novel and more efficient methods for disease control.

1. Introduction

Globalisation and climate change are driving forces that are rapidly changing our world. These unforeseen factors cause rapid expansion of previously controlled or geographically contained parasitic diseases of veterinary importance, such as tick and mosquito borne diseases caused by *Babesia*, *Theileria* and, albeit to a lesser extent, *Plasmodium* (Giles et al., 2014; Karbowiak, 2014; Novikov and Vaulin, 2014; Dantas-Torres, 2015; Tokarevich et al., 2017). In addition, these rapid global changes favour the expansion and importance of other diseases such as toxoplasmosis, neosporosis, eimeriosis, and cryptosporidiosis. The common feature among these diseases is that they are all caused by apicomplexan parasites. If uncontrolled, some of these parasites can severely impact the production of food, but in addition, they can also compromise human health. Other negative consequences include increased acaricide and drug resistance, and the rapid geographical expansion of vectors, parasites and other pathogens by extensive human migration and global transportation of goods and merchandise. These new realities are among the most important and unanticipated public health challenges of the current century and require urgent attention.

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http://dx.doi.org/10.1016/j.ijpara.2017.08.002
0020-7519/Published by Elsevier Ltd on behalf of Australian Society for Parasitology.

Please cite this article in press as: Suarez, C.E., et al. Advances in the application of genetic manipulation methods to apicomplexan parasites. Int. J. Parasitol. (2017), http://dx.doi.org/10.1016/j.ijpara.2017.08.002

The Apicomplexa are a large and diverse ancient phylum of obligate intracellular protozoan parasites that are defined by the presence of an apical complex structure, and include more than 6000 species (Seeber and Steinfelder, 2007). They are arguably the most successful group of obligate parasitic protozoa responsible for life-threatening diseases in domestic, food, and wild animals. In particular, certain apicomplexan parasites, such as *Babesia*, *Theileria*, *Cryptosporidium*, *Eimeria*, and *Toxoplasma* have a high global negative impact on animal health. The global cost of tick transmitted pathogens in the livestock sector alone, of which a significant percentage derives from apicomplexan parasites, was estimated at US $17 billion, approximately 20 years ago (de Castro, 1997). More recently, the global impact of the incidence of neosporosis alone in the cattle industries of 10 selected major countries was estimated to be more than US $1218 million per annum (Reichel et al., 2013). Therefore improved strategies to control these important parasites are clearly overdue.

Apicomplexan parasites typically have complex life cycles and the ability to invade multiple vertebrate and invertebrate hosts and cells thus imposes severe challenges to most currently available control strategies. Their long-term co-evolution with their hosts has produced sophisticated adaptation mechanisms, resulting in parasites that are able to effectively manipulate innate and adaptive host immune responses and, in some cases, transform their host's cells.

Until recently, numerous gaps in our knowledge and understanding of the biology of these parasites and the diseases they cause have severely limited options for designing new methods for control. Research aimed at filling basic key knowledge gaps, including the role of certain genes in the regulation of life stage transitions, the molecular mechanisms involved in host cell invasion, avoidance of host immunity, and parasite transmission, through the application of recently developed gene manipulation methods, will contribute toward the identification of critical parasite-encoded pathways. These research outcomes can be exploited to enhance rational design of novel methods for control, particularly vaccines and drugs.

Given the importance of apicomplexan parasites as pathogens, new research strategies are urgently required to accelerate discoveries leading to improved control. The relatively recent application of a myriad of emerging "omics" disciplines, together with other state-of-the-art molecular biology techniques including biochemical, immunological, and in vitro culture techniques in apicomplexan research, are already enhancing our understanding of the biology of these parasites and creating exciting new hypotheses. In this review, we focus on recent research and possible future directions involving genetic manipulation approaches for *Toxoplasma*, *Neospora*, *Babesia*, *Theileria*, *Eimeria*, and *Cryptosporidium*. *Toxoplasma gondii* is a highly adaptable parasite with the ability to infect a wide range of hosts, and has become a 'model organism' for the Apicomplexa. It can cause abortions in infected animals and has the potential to produce a large zoonotic impact, especially when it infects immunocompromised hosts. *Neospora caninum* is highly related to *Toxoplasma* and also responsible for abortions in livestock. *Babesia* spp. are tick-borne intraerythrocytic parasites that may infect livestock and most domestic animals, causing anaemia and high levels of mortality in naïve adult animals. The tick-borne *Theileria* parasites are unique due to their ability to transform host leucocytes, resulting in high cattle losses in eastern sub-Saharan Africa with severe economic consequences. *Eimeria* includes various species capable of causing coccidiosis in animals, including cattle, sheep, goats, and poultry (Chartier and Paraud, 2012). The more divergent *Cryptosporidium* parasites cause important intestinal disorders in livestock, including diarrhoea, and have high zoonotic potential.

Collectively, this group of apicomplexan parasites is responsible for poorly controlled acute and persistent infections of veterinary importance. Increased strategic and basic research in this field will ultimately lead to enhanced control methods, improved animal health, productivity in crop-livestock and pastoralist systems and, ultimately, human health globally.

2. Basic biological features of apicomplexan parasites

This phylum of parasitic alveolate protozoans comprises obligate endoparasites of animals that share apical complex structures. Apicomplexan parasites are mostly motile, using gliding mechanisms based on myosin motors. The alveolar structure of these protozoans consists of flattened vesicles contained in three membrane layers which are penetrated by micropores. The apical complex provides orientation for specific interactions with host cells, and contains the secretory organelles needed for host cell invasion, including the micronemes (or microspheres in the case of *Theileria*), typically a pair of rhoptries, and dense granules or spherical bodies (Bonnin et al., 1995; Kats et al., 2006; Gubbels and Duraisingh, 2012; Kemp et al., 2013; Swapna and Parkinson, 2017), and other structures such as the conoid (except for the Aconoida *Babesia* and *Theileria* parasites) and polar rings. The apical complex is a secretory structure that is required for invasion of host cells. However, in *Theileria*, the apical complex plays a greater role in establishment after internalisation, rather than invasion. Also 'micronemes' in the strict sense are not discernable by electron microscopy, although 'microspheres' can be observed in these parasites.

Apicoplasts are a chloroplastic remnant derived from a symbiotic organism that invaded a precursor of dinoflagellates and apicomplexans, and are also shared among most apicomplexans (Lim and McFadden, 2010; McFadden and Yeh, 2017). However, plastid DNA has never been identified in *Cryptosporidium*, and it is likely that this parasite lost this organelle in evolutionary history (Sato, 2011).

The genome of apicomplexans is haploid, and the parasites can reproduce asexually by mitosis (merogony and sporogony), and sexually by the fusion of gametes (generated by gametogony, derived from merozoites), concomitant with meiosis occurring in zygotes (Smith et al., 2002). In the case of *Theileria*, and *Babesia*, gamete fusion and meiosis occurs in the arthropod tick vector. These reproductive events may occur in a single host in the case of monoxenous species, such as *Crytosporidium*, or in different hosts, for heteroxenous species such as *Toxoplasma*, *Neospora*, *Theileria* and *Babesia*. The latter develop in definitive hosts, where they undergo sexual reproduction, and in non-definitive hosts by non-sexual mechanisms (e.g. endodyogeny in *Toxoplasma*, merogony in *Theileria* and *Babesia*, and schizogony in *Theileria*) (Smith et al., 2002).

An aspect shared among protozoans, also essential to their pathogenicity, is their ability to disseminate and colonise new hosts. Transmission strategies are also variable among the Apicomplexa. These parasites are diverse and can use distinct modes of transmission including direct (ie: through intimate body contact for example); faecal-oral (e.g., cyst stages of *Cryptosporidium* and *Eimeria*), vector-borne (*Babesia*, *Theileria*, *Plasmodium*) or predator–prey transmission (*Toxoplasma gondii*). Thus, while *Babesia* and *Theileria* parasites are transmissible by ticks and *Plasmodium* via mosquitoes, *Cryptosporidium*, *Eimeria*, *Toxoplasma* and *Neospora* are transmitted via oocysts that are highly resistant to environmental factors.

3. Parasite life cycles

Parasites can have definitive and intermediate hosts. Definitive hosts provide the environment for the sexual reproduction of the

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