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Review

Recombinant anticoccidial vaccines - a cup half full?

Damer P. Blake*, Iván Pastor-Fernández, Matthew J. Nolan, Fiona M. Tomley

Department of Pathobiology and Population Sciences, Royal Veterinary College, University of London, Hawkshead Lane, Hertfordshire AL9 7TA, UK

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ABSTRACT

Eimeria species parasites can cause the disease coccidiosis, most notably in chickens. The occurrence of coccidiosis is currently controlled through a combination of good husbandry, chemoprophylaxis and/or live parasite vaccination; however, scalable, cost-effective subunit or recombinant vaccines are required. Many antigens have been proposed for use in novel anticoccidial vaccines, supported by the capacity to reduce disease severity or parasite replication, increase body weight gain in the face of challenge or improve feed conversion under experimental conditions, but none has reached commercial development. Nonetheless, the protection against challenge induced by some antigens has been within the lower range described for the ionophores against susceptible isolates or current live vaccines prior to oocyst recycling. With such levels of efficacy it may be that combinations of anticoccidial antigens already described are sufficient for development as novel multi-valent vaccines, pending identification of optimal delivery systems. Selection of the best antigens to be included in such vaccines can be informed by knowledge defining the natural occurrence of specific antigenic diversity, with relevance to the risk of immediate vaccine breakthrough, and the rate at which parasite genomes can evolve new diversity. For *Eimeria*, such data are now becoming available for antigens such as apical membrane antigen 1 (AMA1) and immune mapped protein 1 (IMP1) and more are anticipated as high-capacity, high-throughput sequencing technologies become increasingly accessible.

1. Introduction

Eimeria have been recognised as important intestinal parasites of poultry for more than 100 years (Chapman, 2014). During this period understanding of the parasite life-cycle, their global distribution and environmental stability, has become well established (Shirley et al., 2005). Risks associated with uncontrolled coccidial infection include failure of chickens to thrive, increased susceptibility to diseases such as necrotic enteritis, compromised feed conversion and, for some species of parasite, high levels of mortality (Shirley et al., 2005; Williams, 1999). In response, prophylactic anticoccidial drugs are routinely used to control *Eimeria*, and live parasite vaccines are popular in some sectors of the industry (Blake and Tomley, 2014). Diagnosis commonly relies on a combination of pathology (lesion scoring) and detection of oocysts in faeces or litter and has not changed fundamentally in more than 50 years (Nolan et al., 2015). Nonetheless, parasite identification remains largely subjective and differentiation of strains and genotypes is impossible without detailed laboratory analysis. Approaches considered routine for many bacterial pathogens to define drug resistance profiles, or identify the presence of specific virulence factors (Cosentino et al., 2010; Fluit et al., 2001), are not available for *Eimeria*. Oocysts defined by genotypes that confer drug resistance, enhanced virulence or even immunological escape from vaccine-induced protection, appear identical to other oocysts, leaving the farmer, veterinarian and scientist in the dark (Peek and Landman, 2003; Williams et al., 2009). At present such variant parasites are identifiable only by expensive testing *in vivo*, for example determining drug resistance profiles by anticoccidial sensitivity testing (ASTs) (Naciri et al., 2003).

Attempts to develop next-generation recombinant anticoccidial vaccines have led to the identification of many potential vaccine antigens. However, until recently the extent of naturally occurring allelic diversity in the genes encoding these antigens has been unknown (Blake et al., 2015). The relevance of such diversity, and predicting the effects it will have on immune escape and subsequent vaccine failure, is crucial and can help to inform selection of the optimal antigens for inclusion in recombinant vaccine formulations. Pre-existing antigenic diversity in field populations of parasites can immediately limit vaccine efficacy with those expressing allelic variants escaping full control, as has been described in other apicomplexans such as *Plasmodium falciparum* with the antimalarial vaccine candidates apical membrane antigen 1 (AMA1) and merozoite surface antigen 1 (MSP1) (Arnott et al., 2014; Takala and Plowe, 2009). Even where allelic diversity associated with immune escape is low, or occurs/emerges at very low frequency, the selection pressure to survive and replicate in the face of immunity is potent,

* Corresponding author.
E-mail address: dblake@rvc.ac.uk (D.P. Blake).

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favouring genetic diversity and the rapid emergence of resistance.

Selection pressure is particularly profound for *Eimeria* which infect chickens due to a combination of features of the parasite (functionally haploid, highly fecund and rapid replication) and the host (high numbers, intense farming, rapid turnover), as amply demonstrated by the speed with which resistance to chemical anticoccidial drugs emerges (Chapman, 1997). In contrast, live coccidiosis vaccines have been used for more than 50 years with little evidence of parasite evolution towards resistance/immune escape. This is probably attributable to each parasite expressing between 6000 and 9000 antigens during completion of their life-cycle (Reid et al., 2014; Shirley et al., 2005), many of which contribute to robust immune protection. Recombinant vaccines based on small numbers of antigens are likely to exert considerably more focused genetic selection pressure, as is the case for anticoccidial drugs, and parasite genomes will require fewer mutations to achieve immune escape. A key question is whether new anticoccidial vaccines will suffer the same fate as most anticoccidial drugs, with swift selection for vaccine-resistant parasite populations that undermine vaccine value? Without such understanding, the decades of research into recombinant anticoccidial vaccines may be undone within months of vaccine release. In this targeted review we summarise the existing options for control of *Eimeria* species parasites which infect chickens, explore the candidates available for inclusion in sub-unit or recombinant anticoccidial vaccines and discuss the current understanding of genetic diversity for these antigens.

2. Coccidiosis

Eimeria are protozoan parasites of the phylum Apicomplexa. Close relatives of *Toxoplasma gondii* and the *Plasmodium* species, *Eimeria* can cause the disease coccidiosis in all livestock although most species of the genus are strictly host-specific (Kvicerova and Hypsa, 2013; Vrba and Pakandl, 2015). Coccidiosis is a significant economic burden to commercial cattle and sheep production, but the greatest losses occur within the poultry industry, where the disease has been estimated to incur annual deficits in excess of £2 billion (Dalloul and Lillehoj, 2006; Lassen and Ostergaard, 2012; Williams, 1999). Clinical disease manifests as a haemorrhagic or malabsorptive enteritis caused by *Eimeria tenella*, *Eimeria necatrix* or *Eimeria brunetti*, and *Eimeria acervulina*, *Eimeria maxima*, *Eimeria mitis* or *Eimeria praecox*, respectively (Long et al., 1976; Williams et al., 2009). Subclinical infection is common, influencing key production parameters such as food conversion ratio (FCR), average daily gain (ADG), and days to slaughter (Williams, 1999). All *Eimeria* species follow homoxenous faecal-oral life-cycles, offering opportunities for control of transmission in the environment, as well as replication *in vivo*. Husbandry measures including maintenance of dry litter, influenced by variables such as stocking density, quality of housing and ventilation, diet and occurrence of other enteric pathogens, can reduce *Eimeria* transmission, but additional anticoccidial control is essential in modern poultry production.

3. Current anticoccidial control

Ten different active ingredients are currently available in anticoccidial products licensed for prophylactic use with poultry in the European Union, plus one additional therapeutic coccidiocide (http://www.noahcompendium.co.uk/). In the UK between 240 and 300 tonnes of these active ingredients are sold for use in livestock production every year, with the vast majority being used in the poultry sector (Eckford et al., 2014). The drugs available can be divided into chemical and ionophore groups, produced by synthesis or fermentation, respectively (Blake and Tomley, 2014). At present the ionophores dominate the anticoccidial market, representing more than 70% of the drugs used (Eckford et al., 2014), although their status as antibiotics in countries such as the US is beginning to restrict their application. The success of the ionophores has been at least partially attributed to the incomplete anticoccidial protection they provide, even against naïve field isolates, at doses that are not toxic to chickens. This allows the parasite to continue replicating at a low level in the face of treatment (reviewed elsewhere (Chapman, 1999)). For example, in one early study parasite replication and disease pathology caused by apparently susceptible *E. tenella* was not completely blocked in chickens given monensin or lasalocid treatment at 125 ppm. At this dose, equivalent to concentrations used in current commercial applications such as Elancoban® G200 and Avatec® 150G, oocyst output was reduced by 82–97% (monensin), while weight gain was reduced by 10% and 12% (monensin and lasalocid, respectively) and average caecal lesions of 1.2 per group were still recorded (both ionophores) (Chapman, 1976). Subsequently, in trials with monensin at 125 ppm *E. maxima* oocyst output was reduced by 40–92% compared to unmedicated controls (challenge, doses 1000–10 sporulated oocysts per bird, respectively), and *E. brunetti* by 53–98% (Chapman, 1978). Equivalent studies in modern field isolates would be expected to reveal similar or even greater parasite escape as a consequence of drug resistance (Djemai et al., 2016). Furthermore, immunity was found to develop in medicated birds following sequential trickle exposure to *E. maxima*, *E. brunetti* and *E. tenella* (Chapman, 1978). While the comparison between many trials is hindered by experimental variation, low-level parasite escape from ionophore treatment has been a frequent observation (Bafundo et al., 2008; Karlsson and Reid, 1978; Ruff et al., 1980). Indeed, it is accepted that the 'leakiness' of these important anticoccidial drugs is a key factor in their success. Ionophores allow chickens to be exposed to low levels of replicating *Eimeria* that induce natural immune protection that is of huge value to the bird when drugs are withdrawn prior to slaughter, or at the onset of egg production (Chapman, 1976, 1999). Incomplete parasite killing also reduces selective pressure towards drug resistance which probably has extended the active commercial life of the ionophores. Nonetheless, resistance has been described among *Eimeria* to every drug currently available, often appearing within one year of release (Chapman, 1997). Such a swift response against the potent effect of lethal drug selection suggests the occurrence of pre-existing genetic diversity within *Eimeria* genomes and/or a high ability for genome plasticity and diversification.

Anticoccidial vaccination using formulations of live *Eimeria* parasites offers an effective alternative to chemoprophylaxis. Robust immune protection is achieved following ingestion and re-cycling of controlled doses of vaccine oocysts. Anticoccidial vaccine uptake in the poultry industry has been limited by the need for multiple vaccine lines of parasites to be produced by independent passage in chickens. This places some practical limitations on manufacturing capacity and means that vaccines can cost significantly more than anticoccidial drugs (Blake and Tomley, 2014). The first live anticoccidial vaccines such as Coccivac®, and more recently Immucox®, included oocysts of unmodified wild-type *Eimeria* (Williams, 2002). Non-attenuated vaccines are widely used in many parts of the world and offer good vaccine protection; importantly their manufacturing yields are much higher than those of live-attenuated vaccines, so they are considerably cheaper (Chapman and Jeffers, 2014). Because they replicate with high efficiency, live wild-type vaccine parasites also contribute to restoring anticoccidial sensitivity to commercial poultry farms that have drug-resistant populations of *Eimeria*, thus extending the 'life' of several important drugs (Chapman and Jeffers, 2015; Jenkins et al., 2010; Peek and Landman, 2006). However, there are significant drawbacks to the use of non-attenuated vaccines; because the parasites are fully virulent, they carry associated risks of vaccine-induced disease, which has limited their uptake, most notably in Europe where they are not currently licensed. Subsequently, a second generation of live attenuated vaccines was produced, incorporating parasite lines that are selected for early (precocious) development or (in one example) adaptation to growth in embryonic chicks (reviewed in detail by Williams (Williams, 2002)). These second-generation vaccines replicate to lower levels and have superb safety combined with efficacy. However, reduced replication

results in higher vaccine production costs and practical considerations limit the number of doses that can be produced. The success of the first live attenuated vaccines such as Paracox® 8 and Livacox®, particularly for breeding and layer chickens, has stimulated the more recent development of additional attenuated vaccines such as Hipracox®, HuveGuard® and Eimeriavax 4 M®.

For all live coccidiosis vaccines, whether virulent or attenuated, vaccination requires chicks to ingest live oocysts which is achieved *via* direct oral inoculation (rarely used), spraying of vaccine onto chicks where oocysts are ingested by preening, incorporation of the vaccine into edible gel that is distributed to batches of chicks at the hatchery or other similar strategies (Jenkins et al., 2013). Importantly, for most species of *Eimeria* a single round of vaccination does not result in immediate or complete immune protection against a high dose challenge. This requires anywhere between one and three rounds of re-infection (Chapman and Shirley, 2003; Williams, 1998), which is achieved by the re-cycling of vaccine parasites that continues for several weeks with litter oocyst levels normally peaking at between three to five weeks post-vaccination (Williams and Gobbi, 2002). Apart from live oocyst vaccines, the only other coccidiosis vaccine to have reached the commercial market is a killed parasite vaccine, CoxAbic®, which comprises a partially purified preparation of *E. maxima* gametocyte antigens. This product had some success (Sharman et al., 2010) but the requirement for *in vivo* parasite production to prepare vaccine antigen, as well as uncertainty with regards to the need for 'boosting' by exposure to parasites in the field, remain limiting factors (Wallach et al., 2008).

4. Anticoccidial vaccine candidates—how protective to protect?

Live anticoccidial vaccines have proven highly advantageous for use with chickens in the layer and breeder sectors (McDonald and Shirley, 2009) and demand for cheaper subunit or recombinant vaccines appropriate for continuous use in the much larger broiler sector has become intense. Since the early reports of recombinant vaccine development in the 1980s several candidate antigens have been tried and tested by multiple research groups (Blake and Tomley, 2014; Vermeulen, 1998). Small-scale vaccination trials using antigens in recombinant protein, DNA or live-vectored formulations have been reported to achieve 30–90% reductions in parasite replication and/or gut lesion score, or comparable improvements in food conversion ratio and/or body weight gain (*e.g.* Table 1 and Supplementary Table 1). However, despite these encouraging successes no large-scale trials have been reported and considerable variation persists in methodologies and challenge outcomes between laboratories. Additionally, many researchers across both industry and academia continue to search for a 'golden bullet' antigen, or antigen cocktail, that induces complete protection against challenge with an *Eimeria* species parasite. Such a high standard has proven impossible to achieve under experimental or field conditions for apicomplexans such as *P. falciparum* and *T. gondii* (Arnott et al., 2014; Gedik et al., 2016; Takala and Plowe, 2009) and, other than the absence of antigenic switching, there is little reason that *Eimeria* should be any different.

But are we being too stringent? Comparison between current (and even original) ionophore efficacy and live coccidiosis vaccines indicates that comparable levels of partial protection are achieved initially, then this is boosted by subsequent re-cycling of parasites (Williams and Gobbi, 2002). Similarly, formulations of vectored or recombinant vaccine antigens that induce initially incomplete immune protection would also allow limited natural *Eimeria* re-cycling and may subsequently achieve immune protection. Furthermore, the induction of incomplete immunity is likely to reduce the selective advantage of genetic mutations that confer vaccine resistance, improving prospects for long-term vaccine efficacy. According to this rationale, we may already have antigens appropriate for use in combinations as anticoccidial vaccines.

Many *Eimeria* proteins identified as anticoccidial vaccine candidates have roles in host/parasite interaction, probably because these are naturally exposed during parasite invasion/replication and are therefore amenable targets for the host immune response. Among the most widely investigated are proteins secreted from the micronemes, organelles located at the apical tip of apicomplexan parasites whose contents are critical for parasite gliding motility and attachment to host cells, as well as for entry to/exit from infected cells (Rugarabamu et al., 2015). Examples include AMA1, a key component of the parasite-host moving junction (Blake et al., 2011; Blake et al., 2015; Hoan et al., 2014; Jiang et al., 2012), and microneme proteins (MIC)1 (Subramanian et al., 2008; Tomley et al., 1991), MIC2 (Sathish et al.,

Table 1
Examples of the effects of (A) an ionophore and (B) anticoccidial vaccine candidates on parasite replication (percentage reduction in oocyst output by treated *vs* mock- or non-treated chickens). A more detailed summary of anticoccidial vaccine candidates and the experimental conditions applied to their testing are presented in Supplementary Table 1.

(A) Ionophore	Species	Concentration	Reduction (Oocyst dose)	Reference
Monensin	*E. tenella*	125 ppm	82% (100,000)	(Chapman, 1976)
			97% (10)	(Chapman, 1976)
	E. maxima	125 ppm	40% (1000)	(Chapman, 1978)
			92% (10)	(Chapman, 1978)
	E. brunetti	125 ppm	53% (1000)	(Chapman, 1978)
			98% (10)	(Chapman, 1978)

(B) Vaccine candidate	Species	Formulation	Reduction (Oocyst dose)	Reference
Apical membrane antigen 1	*E. maxima*	DNA	42% (250)	(Blake et al., 2011)
	E. tenella	rProtein	66% (300)	(Pastor-Fernandez et al., under review)
gam56	*E. maxima*	DNA	54% (5000)	(Xu et al., 2013)
gam82	*E. maxima*	rProtein	~50% (2000)	(Jang et al., 2010b)
Immune mapped protein 1	*E. maxima*	rProtein	45% (250)	(Blake et al., 2011)
	E. tenella	rProtein	78%, 88% (2000)	(Yin et al., 2013)
Lactate dehydrogenase	*E. acervulina*	rProtein	53% (120,000)	(Song et al., 2010)
Microneme protein 1	*E. tenella*	Yeast	74–79% (6000)	(Chen et al., 2015)
Microneme protein 2	*E. tenella*	rProtein	~38% (10,000)	(Ding et al., 2005)
		Yeast	74% (3000)	(Sun et al., 2014)
Microneme protein 3	*E. tenella*	rProtein	~50% (250)	(Lai et al., 2011)
Profilin (3-1E)	*E. tenella*	rProtein	40% (20,000)	(Lee et al., 2011)
Rhomboid-like protein	*E. tenella*	DNA	76% (30,000)	(Liu et al., 2013)
SO7	*E. tenella*	DNA	~44% (50,000)	(Song et al., 2015c)
TA4	*E. tenella*	DNA	68% (50,000)	(Xu et al., 2008)

2011; Tomley et al., 1996), MIC3 (Labbe et al., 2005; Lai et al., 2011), MIC4 (Tomley et al., 2001; Witcombe et al., 2004), MIC5 (Brown et al., 2000; Zhang et al., 2014), and a putative MIC7 (Huang et al., 2015). Proteins putatively linked to MIC processing such as a rhomboid-like protease (gene ETH_00032220 in the *E. tenella* genome assembly, most similar using comparative homology to *T. gondi* sporozoite-specific protein ROM3 (Shen et al., 2014)) have also shown value as vaccine candidates when delivered in recombinant protein, DNA or *Mycobacterium bovis*-vectored formulations (Li et al., 2012; Liu et al., 2013; Wang et al., 2014; Wang et al., 2009).

Antigen TA4, later identified as the sporozoite-specific glycosylphosphatidylinositol (GPI) anchored surface antigen (SAG)1, one of an extensive set of *Eimeria* surface proteins encoded by multi-gene families, has been widely investigated as a vaccine antigen. SAG1 is able to bind cultured epithelial cells and potentially plays a role in parasite attachment to the host prior to invasion (Reid et al., 2014). SAG1 has been reported to induce partial protective immunity when used as a recombinant protein, DNA or *Salmonella* Typhimurium-vectored vaccine (Brothers et al., 1988; Jahn et al., 2009; Pogonka et al., 2003; Song et al., 2015a; Song et al., 2015b; Song et al., 2009; Xu et al., 2008). More recently, immune mapped protein 1 (IMP1) has been identified as an anticoccidial vaccine candidate, first for *E. maxima* and subsequently for *E. tenella* (Blake et al., 2011; Yin et al., 2015). IMP1 is localised to the sporozoite cell membrane, although its function remains unclear (Jenkins et al., 2015).

Possibly the most widely tested anticoccidial subunit vaccine candidate is profilin, named as 3-1E in many studies (Ding et al., 2004; Min et al., 2001; Song et al., 2000). Profilin is an actin-binding protein involved in microfilament turnover and it is essential in *T. gondii* for parasite gliding motility (Plattner et al., 2008). It is also a ligand for toll-like receptors (TLRs) 5, 11 and 12 which play key roles in initiating immune responses against many intracellular microbial pathogens including *T. gondii* (Koblansky et al., 2013; Plattner et al., 2008; Salazar Gonzalez et al., 2014), suggesting that profilin may act as a novel mucosal adjuvant. Indeed, administration of *Eimeria* profilin enhances the resistance of mice, but not hamsters, to acute phlebovirus infection (Gowen et al., 2006) and increases mouse immune responses to *T. gondii* challenge (Hedhli et al., 2009). Most recently it was shown that *in ovo* administration of combined *Eimeria* profilin, *Clostridium perfringens* NetB protein and Montanide IMS adjuvant provides enhanced protection against necrotic enteritis in an experimental *E. maxima/C. perfringens* challenge model (Lillehoj et al., 2017). Interestingly TLR5 is known to be present in the chicken and is upregulated during *E. tenella* infection (Keestra et al., 2013; Zhang et al., 2012). This led to the testing of flagellin (a TLR5 agonist) as a fusion partner with vaccine candidate *E. tenella* IMP1 with a resultant enhanced protection compared to the use of unfused IMP1 (Yin et al., 2013). Incorporating profilin into this cocktail to further increase TLR5 (and TRR 11 and 12) expression would be an obvious next step.

Subunit vaccine development has also used proteins that are not obviously located exclusively at the host/parasite interface during invasion. Antigen SO7, also named RB1 and GX3262, localises to the sporozoite refractile bodies and induces partial protective immunity when delivered using recombinant protein, DNA or *Salmonella* Typhimurium-vectored strategies (Crane et al., 1991; Klotz et al., 2007; Konjufca et al., 2008; Pogonka et al., 2003; Song et al., 2013; Song et al., 2015a; Song et al., 2015c). Lactate dehydrogenase, used in isoenzyme profiling to differentiate *Eimeria* strains (Shirley, 1978), was shown to induce partial protection against homologous challenge with *E. acervulina*, as well as conferring some heterologous protection against *E. maxima* and *E. tenella* (Schaap et al., 2004; Song et al., 2010). The development of CoxAbic as a subunit vaccine derived from *E. maxima* gametocytes has led to component antigens such as gametocyte antigens gam56 and gam82 to be tested (Jang et al., 2010b; Sharman et al., 2010; Xu et al., 2013).

Several anticoccidial vaccine candidates have been combined with a range of cytokines to adjuvant, and thus enhance, the outcome of vaccination. In the broadest study profilin was tested as a DNA vaccine alongside plasmids expressing interleukin (IL)-1β, IL-2, IL-8, IL-15, interferon (IFN)α, IFNγ, tumour growth factor (TGF) b4 or lymphotactin (Min et al., 2001). The addition of IFNα and lymphotactin were found to improve weight gain during *E. acervulina* challenge, while IL-1β, IL-8, IL-15, IFNy, TGFb4 and lymphotactin decreased parasite replication. However, it was noted that cytokine gene dose and type influenced the quality of the local immune response. Subsequently, co-delivery of IL-2 has been shown to enhance the neutralising antibody response to an infectious bursal disease virus VP2 DNA vaccine (Li et al., 2004). More recently IL-2 has been combined with *Eimeria* antigens such as TA4 (Song et al., 2009; Xu et al., 2008), SO7 (Song et al., 2013) and *E. acervulina* antigen cSZ-2 (Shah et al., 2011), indicating that co-administration of this cytokine can enhance DNA vaccine-induced protective immunity. The CD40 ligand has also been combined with IMP1 to improve antigen presenting cell activation and downstream T-cell-mediated effector functions (Yin et al., 2015). A range of water-in-oil adjuvants have also been used widely in recombinant protein vaccination, including Titermax Gold, Freund's complete and incomplete adjuvants (reviewed in more detail elsewhere (Ahmad et al., 2016)), as well as the Montanide™ series of adjuvants (Jang et al., 2010a).

5. Antigenic diversity with relevance to recombinant vaccines

Understanding the occurrence and extent of genetic diversity across parasite genomes is highly relevant to the study of pathogen persistence and evolution. As selective pressures change, the speed of adaptation can be essential for survival, often influenced by the extent of pre-existing genetic diversity. Levels of intra-specific diversity have been assessed for very few *Eimeria*, but considerable variation has been detected between those species that have been sampled. Comparison of fixation indices (F_{ST}) produced using 248 *Eimeria* ITS1-5.8S-ITS2 sequences including *E. acervulina*, *E. mitis* and *E. tenella* indicated significant intra-specific diversity, with evidence of possible allopatric variation for *E. tenella*, but not the other two species (Clark et al., 2016). More detailed assessment of diversity for *E. tenella* using a genome-wide panel of 52 informative SNPs revealed 93 distinct haplotypes from 244 field samples (flocks), but the calculation of linkage disequilibrium (LD) revealed profound differences between geographical regions (Blake et al., 2015). Most notable were differences in the levels of diversity in *E. tenella* populations from farms in India, where northern parasite isolates presented restricted diversity (eight haplotypes detected across 86 flocks with seven unique to the region) compared to parasites from southern India (50 haplotypes from 53 flocks with 49 being unique). While measures of overall genome-wide diversity and population structure are helpful parameters for predicting the flow of drug- or vaccine-resistant alleles throughout parasite populations, they offer little direct evidence of the likely success of a recombinant anticoccidial vaccine at the time of launch, or over the subsequent years. To assess this, sequencing diversity at the locus which encodes the vaccine candidate(s) is required.

Antigenic diversity has been found to undermine efficacy for many experimental vaccines targeting apicomplexans such as *P. falciparum* (Arnott et al., 2014; Takala and Plowe, 2009). For *Eimeria*, immune escape as a consequence of apparent antigenic diversity has been recognised among strains of *E. acervulina* (Joyner, 1969; Wu et al., 2014), *E. maxima* (Smith et al., 2002), *E. mitis* (McDonald et al., 1985), and *E. tenella* (Awad et al., 2013), although the level of escape is commonly believed to be low in outbred chicken lines. Parasite genetic mapping studies using two strains of *E. maxima* indicate that for this parasite species at least six independent parasite genomic loci are strongly implicated in strain-specific immune protection and/or immune escape, along with additional minor contributions from a large panel of partially protective loci (Blake et al., 2011). Consequently, detailed definition of global diversity in the genes that encode potential vaccine

antigens is a pivotal step towards selection of the most optimal candidates.

To date, detailed sequence analysis to define diversity has been carried out for only a tiny number of candidate vaccine antigens. *Eimeria maxima* AMA1 induces robust protection against homologous challenge when administered as DNA or bacterial-expressed recombinant protein vaccines; it is also a well-studied antigen in other closely related apicomplexan parasites (Arnott et al., 2014; Blake et al., 2011). Using AMA1 to vaccinate chickens under experimental conditions consistently reduces parasite replication (defined here as total oocyst output compared to mock immunised chickens) by 40–80% depending on the vaccination platform used (Blake et al., 2011). Moreover, analysis of the EtAMA1 coding sequence from 56 field samples of *E. tenella*, derived from China, Egypt, Germany, India, Japan, Libya, Nigeria, UK, USA, and Venezuela, revealed a modest level of polymorphism, in striking contrast to the genome-wide diversity described for *E. tenella* (Blake et al., 2015). In total, just seven amino acid isoforms were detected across the 56 sequences. Comparison between countries, and even individual farms, indicated the absence of allopatric selection with multiple AMA1 sequences detected on several farms, and only two found to be constrained by geographical location. Analysis of coding sequence polymorphisms using Tajima's D and Fu and Li′s F* tests of neutrality identified no significant signatures of balancing or directional selection, indicating a largely neutral evolution (Blake et al., 2015). While not sampled to such depth, comparison of *E. tenella* IMP1 coding sequences from parasites in China, India, USA, and UK also indicated a low level of diversity, including expansion/contraction of a CAG triplet repeat and five substitutions, two of which were non-synonymous. As for AMA1, no significant evidence of signatures of selection was detected (Kundu et al., 2017). Low diversity and lack of selection in two key vaccine candidates is in sharp contrast to what has been reported for several leading vaccine candidates in *Plasmodium* species and seems likely to reflect the fact that *Eimeria* parasites have a relatively simple monoxenous life-cycle with direct faecal-oral oocyst transmission, suggesting that there is no requirement for them to persist within the host for an extended period of time. The relatively short prepatent period for *Eimeria* species that infect chickens (between 84 and 138 h, depending on species; ~138 h for *E. tenella* (Long et al., 1976)) is likely to have co-evolved with the speed of the protective host immune response. After primary infection with natural eimerian oocysts, a predominantly T cell-mediated/IFNγ driven adaptive immune response can be detected as early as 96 h (peaking by 144 h post infection for *E. tenella* (Cornelissen et al., 2009; Yun et al., 2000)). The rates of parasite development compared to the timing of expression of protective host immune responses permit completion of the parasite life-cycle in the presence of minimal or incomplete immunity. This suggests a limited role for host immunity in driving selection of natural *Eimeria* antigen evolution, and it is likely to be particularly weak for proteins such as AMA1, which are expressed exclusively during the early (sporogenic) phases of the parasite life-cycle (Blake et al., 2015). Concurrent selection by routine chemoprophylaxis may also reduce signatures of selection.

A broader survey for inter-species signatures of genetic selection comparing non-synonymous to synonymous mutation ratios for 5199 *E. tenella* gene models across orthologues from the other six *Eimeria* that infect chickens indicated pairwise mean K_a/K_s ratios to be 0.24 ± 0.21 (Reid et al., 2014). K_a/K_s ratios of less than 1.0 are indicative of purifying or stabilising selection. Specific K_a/K_s for vaccine candidates such as AMA1 and IMP1, as well as proteins known to be involved in pathogen locomotion, attachment, and/or invasion such as the microneme proteins (MICs) 1–5 and 7, were within the same range or higher than 'reference' genes such as β-tubulin (Fig. 1). This supports the hypothesis that host immunity is not contributing significantly to genetic selection, at least for this subset of genes.

Drivers underpinning the low level of diversity detected for antigens such as AMA1 in *Eimeria* species compared to higher levels of genome-wide diversity are unclear. It is tempting to speculate that rapid genome evolution of an opportunistic parasite that replicates rapidly and exits into an environment where it can survive for extended periods, is likely to be strongly influenced by the swift host turnover in modern chicken production that provides a limitless supply of naïve susceptible hosts. As the parasites complete their endogenous development before the induction of a significant lethal pathogen-specific immune response, there is a reduced drive towards immune-mediated balancing selection. Thus, genes that encode proteins that are essential or important for life cycle progression, for example AMA1 and other invasion-related antigens, could potentially be under selection for optimal biological function, rather than immune evasion, resulting in limited diversity and enhanced prospects for vaccination. Whether correct or not, the limited antigenic diversity discovered for *E. tenella* AMA1 and IMP1 supports the ongoing development of these types of proteins for inclusion in novel anticoccidial vaccines. Further evaluation of antigen-specific selection induced by future subunit vaccination will be required to inform on the likely longevity of efficacy for these vaccines.

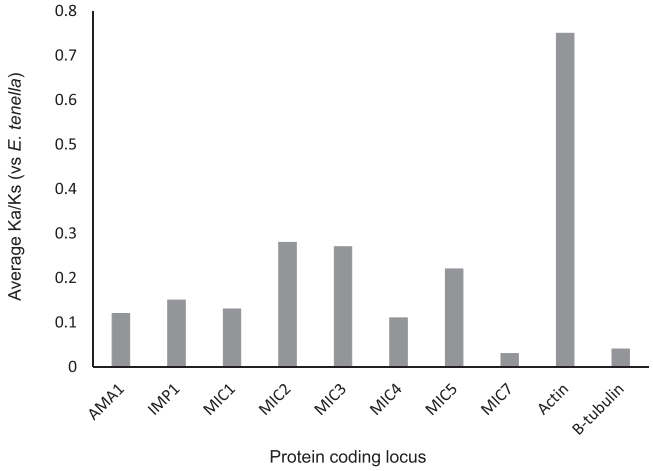

Fig. 1. Average K_a/K_s (non-synonymous/synonymous mutation) ratios for sequences encoding eight anticoccidial vaccine candidates from *Eimeria acervulina*, *E. brunetti*, *E. maxima*, *E. mitis*, *E. necatrix* and *E. praecox*, using *E. tenella* for comparison. Actin and β-tubulin included for comparison. Data derived from Reid and colleagues (Reid et al., 2014).

6. Anticoccidial vaccine antigen delivery—the next big challenge?

The identification of antigens for use in novel anticoccidial vaccines is a significant step towards the development of a novel recombinant vaccine. While experimental monovalent vaccination can induce partial immune protection against individual *Eimeria* species at levels within the lower range of protection achieved by ionophores, it is likely that combinations of two or more antigens will be required to induce protection that is directly comparable. Expanding the concept to multiple *Eimeria* species indicates a requirement for combinations of multiple antigens, preferably combined into a single formulation. The identification of optimal antigen combinations will be driven by considerations of efficacy and safety, and an assessment of pre-existing antigenic diversity should be included in the selection process. It will also be important to determine the nature of the immune responses stimulated by such vaccines, comparing them with those stimulated by natural infection to assess the most effective co-stimulatory molecules and routes of delivery. The delivery of complex multivalent next-generation vaccines remains a massive challenge since multiple individual immunisations are not scalable to the broiler industry. Live replicating vector systems such as transgenic bacteria or parasites offer opportunities for automated single-shot vaccine delivery (Clark et al., 2012; Du and Wang, 2005; Marugan-Hernandez et al., 2016; Tang et al., 2016), while expression in plant or fungal systems can support direct milling of

vaccines into commercial diets for poultry (Chen et al., 2015; Sun et al., 2014; Zimmermann et al., 2009).

7. Conclusions

Several candidate antigens have been identified for use in recombinant anticoccidial vaccines. Levels of protection induced against *Eimeria* challenge using some experimental vaccines have begun to approach those achieved by ionophore-mediated prophylaxis, suggesting that subunit vaccination is now realistic if appropriate combinations can be defined. Consideration of the rather limited genetic diversity found in field populations of *Eimeria* species for candidate antigens such as AMA1 and IMP1 encourages the use of these types of targets for further vaccine development. The inclusion of additional antigens in multivalent formulations is likely to increase the magnitude of immune protection induced as well as delay or prevent the emergence of vaccine-resistant parasite strains in a manner comparable to the use of drug combinations in antibacterial and anticoccidial prophylaxis and therapy (Fischbach, 2011). Further studies with additional candidate antigens and a wider range of *Eimeria* species are now required if optimal multivalent vaccine formulations are to be established and their longevity enhanced.

Abbreviations

ASTs	anticoccidial sensitivity testing
AMA1	apical membrane antigen 1
*Et*AMA1	*Eimeria tenella* AMA1
MSP1	merozoite surface antigen 1
MICs	microneme proteins
IMP1	immune mapped protein 1
SAG1	sporozoite-specific glycosylphosphatidylinositol (GPI) anchored surface antigen 1
FCR	food conversion ratio
ADG	average daily gain
TLRs	toll-like receptors
IFN	interferon
TGF	tumour growth factor
IL	interleukin
Ka/Ks	the ratio of the number of nonsynonymous substitutions/non-synonymous site (Ka), in a given period, to the number of synonymous substitutions/synonymous site (Ks), in the same period

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