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Are *Eimeria* genetically diverse, and does it matter?

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Abstract

Eimeria pose a risk to all livestock species as a cause of coccidiosis, reducing productivity and compromising animal welfare. Pressure to reduce drug use in the food chain makes development of cost-effective vaccines against *Eimeria* essential. For novel vaccines to be successful, understanding genetic and antigenic diversity in field populations is key. *Eimeria* species that infect chickens are most significant, with *Eimeria tenella* among the best studied and most economically important. Genome-wide single nucleotide polymorphism-based haplotyping has been used to determine population structure, genotype distribution, and potential for cross-fertilization between *E. tenella* strains. Here, we discuss recent developments in our understanding of diversity for *Eimeria* in relation to its specialized lifecycle, distribution across the globe, and the challenges posed to vaccine development.

Genome sequences pave new roads for anticoccidial vaccine development

Eimeria species, protozoan parasites that can cause the damaging intestinal disease coccidiosis, pose a significant risk to global poultry production (Dalloul and Lillehoj, 2006, Shirley et al., 2005). Members of the phylum Apicomplexa, the genus *Eimeria* encompasses at least 1 200 species, almost all of which are restricted to a single host (Chapman et al., 2013). Seven *Eimeria* species are recognized to infect the chicken, causing a considerable disease burden across the globe (Fornace et al., 2013, Shirley et al., 2005, Williams, 1998). Similarly, several species are considered to be highly pathogenic in turkeys (reviewed in (Chapman, 2008)). Whilst the pathology associated with each *Eimeria* species infecting chickens has long been understood (Chapman, 2014, Long et al., 1976), parasite population structures and the extent of genetic diversity in field populations are only now emerging. Interest in parasite occurrence, diversity and epidemiology is driven by a global need for cheap and effective vaccines as alternatives to anticoccidial drugs. Details of regional variation in *Eimeria* species prevalence, distribution of genetically and antigenically distinct strains, and the frequency at which polymorphic strains cross-fertilize, all provide valuable knowledge that can underpin rational vaccine design and development. In particular the recent availability of genome sequence resources for all seven *Eimeria* species of the chicken (Reid et al., 2014) provides opportunities to define many of the variables outlined above (Blake et al., 2015b).

Here we review and discuss recent findings relating to the genetic and antigenic diversity of *Eimeria* species which infect chickens in the context of vaccine development and the potential for future successes based on new sequencing technologies and the search for novel vaccine candidate antigens.

52

53 **Current control strategies for *Eimeria* which infect chickens**

54 More than 60 billion chickens are produced in the world every year, yielding
55 1.1 trillion eggs and more than 90 million tonnes of meat (Blake and Tomley, 2014).
56 The poultry industry in the United States of America (USA) alone is worth in excess of
57 $38.1 billion, which includes the combined production value of chickens and turkeys
58 (NASS, 2012). Consequently, effective means of controlling pathogens which infect
59 chickens are essential and of increasing importance as trends for expansion and
60 intensification of global poultry production are maintained (Grace et al., 2012).

61 Control of coccidiosis in poultry relies predominantly on chemoprophylaxis,
62 although resistance to anticoccidial drugs is common in *Eimeria* field populations
63 (Chapman, 1976, Joyner and Norton, 1975, Shirley et al., 2007). Prior to the year 2000,
64 anticoccidial drugs were used in ~95% of flocks where anticoccidial control was
65 employed, including ~99% of commercial broiler flocks (Chapman and Jeffers, 2014).
66 More recently, a study from the USA has reported that this percentage has fallen to
67 between 60 and 99%, depending upon the time of year (Chapman and Jeffers, 2014).
68 While anticoccidial drugs remain essential to chicken production and these trends are
69 not yet reflected in much of the world, reductions in drug application throughout the
70 food chain driven by legislative and consumer pressure is encouraging alternatives for
71 coccidiosis control (Godfray et al., 2010, Shirley et al., 2007). The use of live oocyst
72 vaccines comprising mixes of species of non-attenuated (formerly wild-type) or
73 attenuated parasites (Shirley et al., 2005) are well established. Oral exposure to
74 controlled numbers of vaccine oocysts is designed to result in low grade coccidial
75 infection, inducing a protective immune response that is boosted by re-infection as

76 the live vaccine re-circulates through the chicken house. However, vaccine production
77 costs and the requirement for multiple parasite lines in each vaccine have been
78 significant barriers to the widespread use of live vaccines in the majority broiler
79 production sector (Shirley et al., 2005). Nonetheless, non-attenuated vaccines are
80 now included in anticoccidial rotation programs by 35-40% of commercial broiler
81 companies in the USA (Chapman and Jeffers, 2014).

82 Recombinant subunit vaccines have been considered as potential alternatives
83 for coccidiosis control for many years, and the concept has returned to the fore in the
84 past decade with the discovery and testing of many partially immunoprotective
85 antigens and expansion of the number of vaccine delivery systems available for use in
86 chickens. Low genetic variability in the target antigen(s) is a key requirement for
87 success precisely because recombinant vaccines rely on the expression of a single, or
88 a small number of antigens (Blake et al., 2011, Blake et al., 2004). Vaccination using
89 such a small subset of antigens from a complex parasite such as *Eimeria* may provide
90 a significant driving force for immune selection, which could lead to the rapid
91 appearance and dissemination of alleles which confer vaccine-escape (resistance)
92 (Blake et al., 2015b). The phylum Apicomplexa encompasses a number of parasites
93 important for human and/or animal health including *Plasmodium falciparum* and
94 *Toxoplasma gondii*. The well-characterized population structures and genetic diversity
95 of these parasites have shown that there are numerous barriers to the success of
96 subunit vaccines, but have inspired relevant vaccine development (e.g. (Amambua-
97 Ngwa et al., 2012, Manske et al., 2012, Minot et al., 2012)). In contrast, rather little is
98 known of the genetic diversity and structure of field populations of *Eimeria* parasites,
99 the potential for mixing between genotypes or the selective pressures imposed on loci

which encode immunoprotective antigens, highlighting the numerous challenges posed to the development of novel subunit vaccines (reviewed in (Blake and Tomley, 2014)).

Defining genetic diversity within *Eimeria* species

Eimeria parasites have been recognized for more than a century (Chapman, 2014). Early approaches to understanding parasite diversity focused on parasite (mainly oocyst) morphology, lifecycle (location and timing of development in the gut) and pathogenicity (Tyzzer, 1929). Differences in the mobility of specific metabolic enzymes during starch gel electrophoresis by isoelectric focusing permitted discrimination between *Eimeria* species and some strains (Shirley et al., 1989), but it was only with the application of techniques that visualize DNA such as pulsed field gel electrophoresis to examine chromosomes, and amplified fragment length polymorphism to examine polymorphisms, that genetic variation began to be explored (reviewed in (Beck et al., 2009)). Now, advances in molecular biology permit the detailed definition of genetic diversity at specified loci of interest and across whole genomes (Box 1).

Assessing genetic diversity of *Eimeria* using defined locus sequencing

Sequencing short genomic regions, such as internal transcribed spacer (ITS) or mitochondrial cytochrome oxidase subunit 1 (COX1) loci, has been used widely to infer the relatedness of *Eimeria* isolates, particularly those collected from the field. The technique is relatively inexpensive, can be carried out with limited laboratory resources, and is supported by a published sequence archive with ~1 000 and ~100

124 sequences currently available for ITS and COX1 respectively (GenBank; accessed 7th
125 June, 2016 http://www.ncbi.nlm.nih.gov/pubmed using the 'nucleotide' menu).
126 ITS sequencing has been the molecular technology used most widely for
127 assessing *Eimeria* occurrence in field populations. Initially, studies focused largely on
128 separate countries or continents with examples including Australia, India, Africa and
129 the USA (Cantacessi et al., 2008, Fornace et al., 2013, Godwin and Morgan, 2015,
130 Kundu et al., 2015, Schwarz et al., 2009). The most comprehensive survey of *Eimeria*
131 field isolates was published recently in which 512 pooled faecal samples were
132 surveyed from poultry farms situated in 20 countries across five continents (Clark et
133 al., 2016). Here, ITS1-5.8S-ITS2 sequence analysis revealed some interesting aspects
134 of population structure. The genetic signatures of *Eimeria acervulina* and *Eimeria mitis*
135 indicated that regular interbreeding occurs between genotypes, while *Eimeria tenella*
136 exhibited a more restricted population structure (Blake et al., 2015a, Clark et al.,
137 2016). The inclusion of sequences derived from laboratory reference strains that are
138 progenitors to many vaccine parasites in the comparison suggested that the samples
139 collected were representative of wild-type field strains, not re-sampling of vaccinal
140 lines [31]. It was suggested that the faster generation time and greater fecundity of *E.*
141 *acervulina* and *E. mitis* compared to *E. tenella* (~33% shorter prepatent period and 2.5-
142 4 times more oocysts produced per oocyst ingested (Bumstead and Millard, 1992,
143 Eckert et al., 1995)) could account for the observed differences in population
144 structure. As a consequence, *E. acervulina* and *E. mitis* parasites have greater
145 opportunity for co-infection and hybridization and their genomes may evolve more
146 rapidly.

147 Analysis of ITS sequence datasets has also led to the discovery of three new
148 *Eimeria* 'operational taxonomic units' (OTUs) (Cantacessi et al., 2008, Clark et al.,
149 2016, Fornace et al., 2013, Godwin and Morgan, 2015). Initially, ITS2 sequencing of
150 isolates from Australia provided the first definition of the three *Eimeria* OTU
151 genotypes termed OTUx, OTUy and OTUz (Cantacessi et al., 2008), which have been
152 supported by subsequent ITS1 and ITS2 sequencing of isolates covering a greater
153 geographical range (Clark et al., 2016, Godwin and Morgan, 2015, Jatau et al., 2016).
154 These divergent parasites appear to be restricted at present to southern regions of
155 the world below 30^{o}N latitude (Clark and Blake, 2012, Clark et al., 2016), although
156 future human and trade movements risk the expansion of their range. The spread of
157 parasites with these novel genotypes may have significant consequences for vaccine
158 development and application. At present it is unclear whether these variants can
159 evade the immune protection offered by live vaccines, although Morris and colleagues
160 have provided one example of escape from the field (Morris et al., 2007). Sequence
161 comparison currently suggests that OTUx is most closely related to *Eimeria maxima*,
162 with *Eimeria brunetti* the closest link to OTUy (Godwin and Morgan, 2015).
163 Comparison of ITS1-5.8S rDNA-ITS2 sequences has revealed the greatest divergence
164 for OTUz with distinct long and short forms, as described previously for *E. maxima* and
165 *E. mitis* (Clark et al., 2016, Schwarz et al., 2009).
166 The development of next generation sequencing technologies has moved
167 analysis of genomic diversity from the single gene to genome wide levels, vastly
168 increasing available genetic and genomic resources for *Eimeria*. For example, fully
169 resolving the phylogenetic relationships between *Eimeria* species which infect
170 chickens and turkeys has proven difficult based on COX1 and 18S rDNA sequences

171 alone (El-Sherry et al., 2013, Miska et al., 2010, Ogedengbea et al., 2011). The
172 robustness of separation of the seven *Eimeria* species recognized to infect chickens
173 was greatly improved using whole-genome phylogenies (Reid et al., 2014) and may
174 prove beneficial in future analyses of field isolates. Mitochondrial genome sequencing
175 has also been used effectively to separate *Eimeria* species which infect domestic
176 turkeys (Ogedengbe et al., 2014a) (Table 1). The addition of genome sequences
177 resources for cloned OTU x, y and z lines are a high priority and should resolve the
178 cryptic status of these genotypes.
179
180 **A Dynamic and Adaptable Genome?**
181 Beyond the resolution of phylogenetic debate, whole genome sequencing has
182 revealed interesting aspects of genome structure for *Eimeria* (refer to Table 1 for a
183 summary of resources). Initial analysis of *E. tenella* chromosome 1, sequenced
184 following purification from pulse field gel electrophoresis-resolved karyotypes,
185 revealed alternating regions of repeat-poor (P) and repeat-rich (R) sequences (Ling et
186 al., 2007). More recently, Illumina-based genome sequencing and assembly
187 demonstrated that the P and R structure was not limited to chromosome 1, but was
188 conserved in all chromosomes of *E. tenella*, and across the genomes of *E. acervulina*,
189 *E. brunetti, E. maxima*, *E. mitis*, *E. necatrix*, and *E. praecox*, as well as *Eimeria*
190 *falciformis* (a parasite specific to the mouse) (Heitlinger et al., 2014, Reid et al., 2014).
191 Interestingly, differences were observed in repeat content between species. *Eimeria*
192 *tenella*, for example, has fewer R regions than the other six species which infect
193 chickens, while *E. necatrix* was more repeat rich across its genome, most notably in
194 regions syntenic with *E. tenella* (Reid et al., 2014). This P/R structure does not appear

195 in the genomes of other coccidia such as *Neospora caninum* or *T. gondii* (Reid et al.,
196 2014, Reid et al., 2012), although it has been detected in the more closely related
197 *Cyclospora cayetanensis* genome (Liu et al., 2016). Ling and colleagues have suggested
198 that the unusual genome organization might pose an evolutionary advantage to the
199 parasite by facilitating rapid evolution and diversification. Variation in restriction
200 fragment length polymorphism (RFLP) fragment size between different strains of *E.*
201 *tenella* associated with R-, but not P-regions, lending some support for genome
202 plasticity (Ling et al., 2007, Reid et al., 2014, Shirley, 1994). A disproportionately high
203 repeat content in protein coding sequences could confer some evolutionary
204 advantage, although their effects on protein structure appear to be neutral and genes
205 known to be integral to host–parasite interaction were relatively free of repeats (Reid
206 et al., 2014). The seven *Eimeria* species which infect poultry do not appear to possess
207 the sub-telomeric regions which in *P. falciparum* contain a set of plastic genes involved
208 in host immune system evasion (Gardner et al., 2002, Ling et al., 2007, Reid et al.,
209 2014). Telomere-like repeats are, however, dispersed throughout the R-segments,
210 suggesting there are complexities in the structure of the genome that we do not fully
211 yet understand. Telomere-like repeats have previously been described in the
212 *Plasmodium knowlesi* genome where they associate with variant antigen families,
213 although a similar linkage has not been described for *Eimeria* (Pain et al., 2008). The
214 impact of the segmented *Eimeria* genome structure on the appearance and extent of
215 genetic diversity is yet to be determined, although it may well associate with hotspots
216 of genetic recombination. The implications of such hotspots on vaccine development
217 are similarly unclear.
218

The importance of population structure

Genetic mapping has been useful in establishing the population structure of some apicomplexans (reviewed in (Clark and Blake, 2012)), as have other molecular tools (reviewed in (Beck et al., 2009)). Population structure varies across the Apicomplexa. *Plasmodium falciparum* has been shown to exhibit signatures of panmictic or clonal population structures, influenced in part by regional transmission rates (Annan et al., 2007, Larranaga et al., 2013). *Toxoplasma gondii* is commonly clonal in much of the world, with a small number of dominant genotypes described, although a higher level of genetic diversity has been detected in regions such as South America where population mixing appears to occur at a greater frequency (Minot et al., 2012, Su et al., 2012). For *E. tenella*, comparison of haplotype occurrence and diversity defined following multiplex single nucleotide polymorphism (SNP) genotyping revealed that north Indian and north African field populations were characterized by a limited number of distinct haplotypes and significant linkage disequilibrium (Figure 1), resembling the region specific population structure of *T. gondii* (Blake et al., 2015b). This population structure suggests that limited opportunities exist for cross-fertilization and genetic recombination, and that the expansion of a small number of haplotypes might be common although not necessarily clonal. In contrast, a greater haplotype diversity was reported in southern India and Nigeria with multiple haplotypes appearing, all at a very low frequency, indicating that co-infection with heterologous isolates and cross-fertilization is common during sexual reproduction, and that genetic diversity is likely to be greater than estimated by current sampling (Blake et al., 2015b). These findings suggest there are numerous opportunities for recombination in the field in these regions.

243 The regional differences in population structure observed for *E. tenella* may
244 have several underlying causes. In southern India there is a greater poultry density
245 than found in the north (Grace et al., 2012), and therefore more opportunity for
246 parasite co-infection and cross-fertilization. Further, the climate in south India is
247 commonly more humid than in north India (Deichmann and Eklundh, 1991), likely
248 favoring higher levels of oocyst sporulation and increased parasite survival in the
249 poultry house environment as reported in comparisons of rainy versus dry seasons
250 (Awais et al., 2012). Higher rates of transmission commonly associate with elevated
251 levels of outcrossing and increased genotype abundance for other apicomplexans
252 such as *P. falciparum* (Larranaga et al., 2013). Importantly, co-infection of a single host
253 with two or more genetically distinct *Eimeria* isolates does not guarantee cross-
254 fertilization. The *Eimeria* life cycle includes a single, transiently diploid phase during
255 sexual reproduction and oocyst maturation (Walker et al., 2015), so timing of the co-
256 infection has to be essentially simultaneous for genetic recombination to occur.
257 Additionally, the *in vivo* phase of the *Eimeria* life cycle is predominantly self-limiting,
258 with features such as prepatent period and the number of rounds of schizogony
259 stable, unless subjected to deliberate selection for developmental rate (Blake et al.,
260 2015b, Lal et al., 2009, Shirley and Harvey, 2000). Studies using major
261 histocompatibility complex (MHC) class I or II knockout mice suggest little or no role
262 for the host immune response in the conclusion of parasite replication (Smith and
263 Hayday, 2000). Thus, gametes of each genotype must mature in parallel for cross-
264 fertilization to take place. *In vivo* experiments using laboratory strains of *E. tenella*
265 have shown that, given the opportunity, cross-fertilization is common, highlighting the
266 potential that *E. tenella* has to hybridize in field populations and indicating the ease

with which vaccine or drug-resistant alleles could propagate in field parasite populations (Blake et al., 2015b). Combined, these factors emphasize the importance of considering region specific environmental and social variables in implementation of novel control strategies for *Eimeria* species. Fornace and colleagues demonstrated that the diversity of species present in small-scale production systems in Africa was directly linked to profitability (Fornace et al., 2013). However, there have been few similar studies and the potential is there to link population structure and the burden of coccidiosis to profitability in particular regions of the globe.

The relevance of antigenic diversity

Selection of candidate antigens for vaccine development has proved to be a significant barrier to progress in other Apicomplexa such as *T. gondii* and the *Plasmodium* species (Alexander et al., 1996, Liu et al., 2012b, Stanisic et al., 2013). Differentiating immunogenicity from 'true' immune protection can be difficult, making selection of protective antigens problematic (Blake et al., 2011). In one example, homologs of apical membrane antigen 1 (AMA-1) have been shown to be protective in a range of apicomplexan parasites including *E. maxima* (Blake et al., 2011), *E. tenella* (Jiang et al., 2012) and *P. falciparum* (Drew et al., 2012, Eisen et al., 2002, Healer et al., 2004), and it has been widely proposed as a candidate for subunit vaccine development. However, extensive allelic diversity has limited development of *P. falciparum* AMA-1, with more than 60 polymorphic amino acid residues detected and more than 200 haplotypes within even a single population (Drew et al., 2012, Healer et al., 2004, Hodder et al., 2001, Terheggen et al., 2014). Despite such discouraging reports from *P. falciparum*, AMA-1 has shown promise as a vaccine

291 candidate for *E. tenella*, with a potent inhibitory effect on parasite invasion (Jiang et
292 al., 2012). More recently, genotyping *E. tenella* field isolates collected from Africa and
293 India suggested that polymorphisms in the EtAMA-1 locus are lower than expected in
294 field populations with largely neutral signatures of selection. The functionality of
295 AMA-1 may outweigh the potential benefit to the parasite of immune evasion, which
296 may be of limited value in the self-limiting eimerian life cycle (Blake et al., 2015a).
297 Similarly, just four nucleotide polymorphisms exist between EmAMA-1 coding
298 sequences from the *E. maxima* Houghton and Weybridge laboratory strains, two
299 causing non-synonymous changes, one situated in the putative pro-domain and one
300 located in domain 1 (Blake et al., 2012, Reid et al., 2014). Nonetheless, despite such
301 limited diversity within the coding region of at least one vaccine candidate, strain
302 specific immune escape has been reported *in vivo* for *E. acervulina* (Joyner, 1969, Wu
303 et al., 2014), *E. mitis* (McDonald et al., 1985), *E. maxima* (Smith et al., 2002) and *E.*
304 *tenella* (Abu-Akkada and Awad, 2012, Awad et al., 2013, Fitz-Coy, 1992). Comparison
305 of *E. tenella* isolates collected from chickens reared in British and Indian poultry
306 houses revealed incomplete immune protection between isolates, most notably
307 following low-level primary exposure (Blake et al., 2015b). Despite these reports,
308 there is no evidence that vaccine resistance has evolved in response to whole live
309 parasite vaccination (Blake and Tomley, 2014, Shirley et al., 2005). One possible
310 explanation for this is that throughout its lifecycle each *Eimeria* species expresses
311 between 6 000 and 9 000 proteins (Reid et al., 2014), exposing the host to a complex
312 portfolio of antigens. Selection targeting multiple immunoprotective antigens in
313 parallel during replication in the chicken is likely to limit the capacity for any individual
314 parasite to evade the host immune response as a consequence of diversifying

315 selection. Thus, the complexity of the antigenic repertoire might explain why
316 resistance to live parasite vaccination has not yet developed (Blake et al., 2015a, Blake
317 and Tomley, 2014). Incorporating multiple antigens, in addition to AMA-1, in novel
318 subunit vaccines would therefore be likely to extend their potential for long term
319 success by buffering the effects of diversifying selection on a single target antigen.
320
321 **Life cycle stage-specific antigen expression and immune selection**
322 Each *Eimeria* life cycle features a series of extra- and intracellular stages within
323 the definitive host as the parasite undergoes successive rounds of asexual, and then
324 sexual replication (Reid et al., 2014, Walker et al., 2015). Throughout this process
325 *Eimeria* expresses many of its genes in a stage-specific manner which can impact on
326 the development of novel vaccines. In *T. gondii*, for example, vaccination with life cycle
327 stage-specific antigens leads to stage-limited protection (Alexander et al., 1996, Liu et
328 al., 2012b). In *Eimeria*, the early life cycle stages are important to the induction of
329 protective immunity during natural infection (Blake and Tomley, 2014, Jiang et al.,
330 2012, Reid et al., 2014). Importantly, vaccine candidates such as AMA-1 are primarily
331 expressed by a single life cycle stage and are unlikely to be subjected to a protein-
332 specific adaptive immune response during primary infection given the absence of
333 protracted colonization (Blake et al., 2015a, Jiang et al., 2012, Lal et al., 2009). Thus,
334 the large oocyst output resulting from even low dose primary infections results in
335 considerable environmental contamination with parasites which have never been
336 exposed to immune selection.
337
338 **Future directions**

A clear direction for future work is to expand our understanding of population structure to other *Eimeria* species in the field as has been reported recently for *E. tenella* (Blake et al., 2015a). Elucidating the population structure and potential for mixing is key in the development of novel control strategies for *Eimeria*. Understanding the possible biological, environmental, industrial and social drivers which underpin the observed diversity may be even more important, demanding detailed epidemiological interrogation. Opportunities to develop medium/high throughput tools such as Sequenom-based genotyping, and new high-throughput sequencing technologies such as restriction site associated DNA (RAD) sequencing, will facilitate the move away from ITS sequencing to genome wide analysis of genetic diversity with particular relevance to field samples. *Eimeria* genomic resources have increased greatly in recent years (reviewed in (Blake, 2015)). Additionally, since the cost of sequencing a genome the size of *E. tenella* is now relatively modest (51.8 Mb DNA in the current genome assembly (Reid et al., 2014)), the opportunity exists to build on the available genomic resources with whole genome sequencing of other *Eimeria* strains and species. Parasites of the three OTU genotypes are obvious candidates, with species which infect other livestock species further priorities. The genomes of non-target species can yield clues as to the structure and function of other closely related species. Comparative analysis of the *E. falciformis* genome with *T. gondii* revealed a shared emergence and diversification across the Coccidia of gene families associated with motility and invasion (Heitlinger et al., 2014). Building on information from whole genome sequencing, another relatively new technology, RNA sequencing (RNASeq) can be used for transcriptomic profiling of other key antigens of interest and is likely to offer clues as to their function and suitability as vaccine targets.

363 RNASeq has already been used successfully to define transcriptomes from several
364 *Eimeria* life cycle stages (Reid et al., 2014, Walker et al., 2015). Indeed in the near
365 future Isoform sequencing (IsoSeq), which at present generates transcripts >3Kb
366 (Tilgner et al., 2014), could be utilized to sequence the entire transcriptome of a single
367 parasite in full length fragments. Genome editing techniques such as the CRISPR/Cas
368 system have huge potential and could be used, for example, to switch allelic type for
369 a small number of target antigen coding genes. The CRISPR/Cas system has been used
370 successfully in *P. falciparum* (Ghorbal et al., 2014, Wagner et al., 2014) and *T. gondii*
371 (Shen et al., 2014, Sidik et al., 2014), but is not yet available for *Eimeria*. These tools
372 should improve the molecular definition of diversity, expand our understanding of
373 parasite evolution and host evasion, and highlight regions of the genome that show
374 promise in the development of novel sub-unit vaccines.
375
376 **Concluding Remarks**
377 There are several key challenges posed by population, genetic and antigenic
378 diversity of *Eimeria* parasites to the development of novel vaccines (see Outstanding
379 Questions). How genetic, particularly antigenic, diversity influences pathogenicity,
380 vaccine specificity and epidemiology, and the implications of this for effective
381 intervention and control, are important questions that need to be answered for all
382 apicomplexan parasites. Recent studies have revealed a polarized global occurrence
383 for genetically divergent *Eimeria* strains, and possibly even new species, that may be
384 capable of replicating within chickens vaccinated using current generation vaccines.
385 These parasites pose a significant risk to vaccine efficacy, and thus food security and
386 animal welfare, in production systems which rely on anticoccidial vaccination.

Considering social and environmental variables in novel control strategies is of great importance, with factors including choice of production system, geographic separation of farms and climatic conditions likely to influence parasite population dynamics. The recent expansion in genetic and genomic resources available for *Eimeria* has dramatically improved our ability to genotype parasites recovered from field populations and begin to assess how many of these variables will affect genetic diversity, and whether that diversity will impact on vaccine efficacy and longevity.

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696 **Figure legends**

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698 **Figure 1. Median-joining phylogenetic NETWORKs illustrating genome-wide and**
699 **antigen specific diversity reported for *Eimeria tenella*.** A. The influence of geographic
700 origin on *E. tenella* single nucleotide polymorphism (SNP) haplotype occurrence and
701 complexity. Parasite populations from Nigeria and south India presented high
702 haplotype diversity and apparent panmixia, compared to more restricted variation in
703 north African and north Indian populations. Node size indicates the frequency of
704 haplotype occurrence. Figure reproduced from (Blake et al., 2015b). B. Coding
705 sequence polymorphism within the apical membrane antigen 1 (AMA-1) locus. Eight
706 allelic types were detected with less geographic specificity than described for genome
707 haplotypes. Figure derived from data presented in (Blake et al., 2015b).

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713 **Table 1**. **Genome size and available genetic resources for *Eimeria* species in comparison with the apicomplexan species**
714 ***Toxoplasma gondii* and *Plasmodium falciparum*[a].**
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Species	Host	Genome Size (Mb)	Reference Genome	Mitochondrial Genome	RNASeq	Defined Locus Sequencing	SNP Arrays	Proteomics
Eimeria falciformis	Mouse	43.67	●			●		
Eimeria acervulina	Chicken	45.83	●	●		●		
Eimeria brunetti	Chicken	66.89	●	●		●		
Eimeria maxima	Chicken	45.98	●	●		●		
Eimeria mitis	Chicken	72.24	●	●		●		
Eimeria necatrix	Chicken	55.01	●	●		●		
Eimeria praecox	Chicken	60.08	●	●		●		
Eimeria tenella	Chicken	51.86	●	●	●	●	●	●
Eimeria adenoeides	Turkey	-		●		●		
Eimeria dispersa	Turkey	-		●		●		
Eimeria gallopavonis	Turkey	-		●		●		
Eimeria innocua	Turkey	-		●		●		
Eimeria meleagridis	Turkey	-		●		●		
Eimeria meleagrimitis	Turkey	-		●		●		
Toxoplasma gondii GT1	Cat, others	63.95	●	●	●	●	●	●
Plasmodium falciparum	Mosquito, human	23.3	●	●	●	●	●	●

716 [a]Table adapted from the *Toxoplasma* Genetics Resource *ToxoDB* (http://www.toxodb.org/toxo/showApplication.do, accessed 15th June,
717 2016), supplemented by (Hafeez et al., 2016, Hikosaka et al., 2011, Hnida and Duszynski, 1999, Lin et al., 2011, Liu et al., 2012a, Ogedengbe
718 et al., 2014b, Ogedengbe et al., 2013, Vrba and Pakandl, 2014).

719 [b]● = sequence resource available.

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Box 1 – The utility of SNP genotyping assays in determining parasite population dynamics in the field.

Defined locus sequencing has been widely used to genotype *Eimeria* field isolates since it is cost effective and relatively quick to accomplish. However, with the advent of new high throughput sequencing technologies analysis of genetic diversity across whole genomes is now possible. When whole genome sequencing first became available the associated costs were prohibitive, but this is changing rapidly as the technology becomes cheaper. Reference genome sequence assemblies are now available for the seven *Eimeria* species that infect chickens (Reid et al., 2014). Large-scale genome re-sequencing of field isolates will soon be possible but is not yet affordable. In the interim period sequencing a small number of additional strains for comparison with the relevant reference genome provides a resource to design genotyping tools based on specific single nucleotide polymorphisms (SNPs). Custom SNP-based assays are a cost effective method of genotyping parasites which can be applied effectively to large-scale collections of field isolates (Blake et al., 2015a). SNP genotyping technologies provide useful tools to assess the level of cross-fertilization and genetic recombination in field populations. Such knowledge can be employed to improve the prospects of future subunit vaccines being effective in the field.