

1 **Securing poultry production from the ever-present *Eimeria* challenge**

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14 **Keywords:** Coccidiosis, vaccines, chickens, food security, new approaches

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17 **Abstract**

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19 The intestinal disease coccidiosis, caused by protozoan parasites of the genus *Eimeria*, is
20 one of the most important livestock diseases in the world. It has a high impact in the poultry
21 industry where parasite transmission is favoured by high-density housing of large numbers
22 of susceptible birds. Coccidiosis control in poultry is achieved by careful husbandry
23 combined with in-feed anticoccidial drugs or vaccination with live parasites. However,
24 outbreaks of coccidiosis still occur and sub-clinical infections, which impact significantly on
25 productivity and food security, are common due to widespread drug resistance, high parasite
26 prevalence, and environmental persistence. Herein, we review some recent approaches for
27 the production of cheaper third generation vaccines, based on robust methods for
28 identification of immunoprotective antigens and the use of transgenic *Eimeria*.

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46 **Sustainable poultry production in the face of *Eimeria* challenge**

47 Global poultry production has tripled in the last 20 years and the world's chicken flock is
48 estimated at approximately 21 billion, producing 1.1 trillion eggs and approximately 90
49 million tonnes of meat (equivalent to ~ 60 billion carcasses) each year (www.faostat.fao.org).
50 Expansion is predicted to continue for at least 30 years with Africa and Asia accounting for
51 the most growth [1]. Commercial poultry production is possible only with the support of
52 effective pathogen control, including good animal husbandry, chemoprophylaxis, and
53 vaccination. A major and recurring problem is coccidiosis [2-4], an enteric disease caused by
54 protozoan *Eimeria* species (see Glossary), which are parasitic coccidians with homoxenous
55 faecal-oral life cycles (Figure 1) that are closely related to *Toxoplasma*, *Neospora*, and
56 *Isospora* and more distantly related to other apicomplexans including *Babesia*, *Plasmodium*,
57 and *Theileria* [5, 6]. Seven species of *Eimeria* infect the chicken with absolute host-
58 specificity, causing haemorrhagic (*Eimeria brunetti*, *Eimeria necatrix*, and *Eimeria tenella*) or
59 malabsorptive (*Eimeria acervulina*, *Eimeria maxima*, *Eimeria mitis*, and *Eimeria praecox*)
60 disease. These parasites are highly prevalent and can persist for long periods in the
61 environment, including in faeces and litter (bedding/substrate). Thus, most chicken flocks in
62 the world are exposed and many chickens become infected. Uncontrolled outbreaks cause
63 high morbidity and mortality, and if infections are only partially controlled then sub-clinical
64 disease is common and economically relevant because it causes poor feed conversion,
65 reduced egg production, and failure to thrive. Comparison of the costs incurred by veterinary
66 infectious diseases in the UK has highlighted coccidiosis as a leading problem in terms of
67 total cost, including the cost of control [7]. The global economic impact of coccidiosis is
68 unclear but has been estimated to be in excess of \$3 billion USD per annum due to
69 production losses combined with costs of prevention and treatment [8, 9]. Additional risk has
70 been noted in much of the developing world where *Eimeria* can undermine the rapid
71 expansion of poultry production and exert a profound impact on local poverty [10]. Costs in
72 other livestock sectors where *Eimeria* parasites are also prevalent are less well defined but
73 likely to be similarly high [11]. Links between eimerian infection and increased intestinal
74 colonisation of bacterial pathogens such as *Clostridium perfringens* and *Salmonella enterica*
75 serovars Typhimurium and Enteritidis increases risk to food security and raises concerns of
76 zoonotic food-borne disease [12-14]. The global distribution of the *Eimeria* species,
77 complemented by their ability for environmental survival as oocysts and their propensity for
78 drug resistance, poses a serious threat to secure production of poultry-derived food
79 products. The control of *Eimeria* remains as important now as it has ever been with the
80 development of cost-effective, scalable vaccines required urgently.

81

82 **Current options for control**

83 Successful commercialisation of poultry production in housed, free-range, and organic
84 systems relies on effective control of *Eimeria* parasites. Good husbandry plays a key role in
85 limiting oocyst sporulation and recycling through measures such as restricting bird access to
86 faeces, maintaining litter quality, controlling house temperature, ventilation, and moisture
87 levels, and thorough cleaning between flocks. However, husbandry alone is inadequate for
88 control, and prophylactic anticoccidial drugs (ionophores and other chemicals) are crucial for
89 sustainability of most production systems. In the UK more than 40% of all antimicrobials
90 sold for use in food and non-food animals are classified for the control of coccidial parasites
91 (277 tonnes of active ingredient in 2011; mostly for control of *Eimeria*) with ionophores
92 representing more than 70% of these [15]. Resistance to anticoccidial drugs has been
93 recognized for decades and regarded as ubiquitous [16] with wide acceptance that drugs
94 remain effective in the field only because they suppress parasite growth sufficiently to allow
95 birds to develop natural immunity [17]. The speed at which resistance can appear, combined
96 with legislative restrictions on the use of many in-feed drugs in some countries and
97 consumer concerns regarding chemical residues in food products has discouraged most
98 attempts to develop new anticoccidials.

99

100 The major alternative option for coccidiosis control is vaccination, which is used effectively to
101 protect egg-laying and breeder chickens but applied more rarely within the majority broiler
102 sector. Anticoccidial vaccines are available in some countries for use with turkeys (for
103 example Coccivac-T®), but vaccines targeting *Eimeria* species that parasitise mammals are
104 yet to be commercialized. With one exception, all of the anticoccidial vaccines that are
105 currently available are based on varied formulations of multiple live species of *Eimeria*. The
106 development and compositions of these vaccines have been reviewed thoroughly in recent
107 years and will not be described in depth here [3, 8, 18]. Briefly, live anticoccidial vaccines fall
108 into two major groups. The first generation, based on formulations of live wild-type parasites,
109 includes vaccines such as Coccivac®-B and -D and Immucox®, all of which are still currently
110 available. First introduced in 1952, live wild-type vaccines rely on the controlled exposure of
111 chickens to small numbers of virulent oocysts that initiate infection and induce a natural
112 protective immune response. While effective and still widely used in some sectors of the
113 poultry production industry [3], these vaccines have a requirement for “even” vaccine
114 distribution across the flock to avoid occurrence of disease. Ingestion of too-high a dose can
115 cause a direct negative impact on feed conversion or even clinical symptoms of
116 haemorrhagic or malabsorptive coccidiosis, while ingestion of too-small a dose leaves birds
117 unprotected against large numbers of recycled vaccine oocysts excreted by other flock

118 members under anything but the very best flock management [19]. Recognition of the risk
119 posed by vaccinal pathogenicity prompted development of second generation live
120 attenuated vaccines. Most of these vaccines contain parasites derived by selection for the
121 trait of “precocious” development (i.e., more rapid completion of the life cycle with a reduced
122 pre-patent period, lower reproductive capacity and consequential reduction in pathogenicity).
123 Parasite lines selected through multiple rounds of *in vivo* passage to be capable of
124 completing their life cycles 13-33 hours faster than their wild-type progenitor retain full
125 immunoprotective capacity while losing one, or even two, rounds of schizogony [20, 21],
126 making them safer, and attenuated vaccines. A small number of attenuated lines have also
127 been developed by serial passage of parasites through embryonating eggs [21-23], a
128 process that results in parasites that are significantly less pathogenic for chickens than their
129 wild-type progenitor [24]. Second generation vaccines were introduced nearly 40 years after
130 the first wild-type vaccines and include Paracox® (attenuated by selection for precocity,
131 registered 1989) and Livacox® (attenuated by selection for precocity or egg adaptation,
132 registered 1992), which remain highly successful with > 1 billion doses of the former sold
133 each year [25]. The marketing of these major second generation products has in turn
134 stimulated the generation of several more attenuated lines from parasites around the world
135 as the basis of regional vaccines (e.g., [26-28]). These successes have been tempered,
136 however, by inherent production limitations. *Eimeria* parasites cannot yet be propagated
137 efficiently *in vitro* [29, 30], which means all vaccine lines have to be grown in chickens. The
138 relatively low reproductive index of attenuated compared to wild-type vaccine lines increases
139 production, and thus retail, costs (retail between UK £0.04 and £0.30 per dose depending on
140 formulation; <http://www.animal-meds.co.uk/>), and perhaps more importantly imposes
141 practical limitations on the number of doses that can be produced. For these reasons, live-
142 attenuated vaccine usage is confined mainly to the breeding and egg-laying sectors of the
143 poultry industry. Attempts to develop live vaccines attractive to the broiler sector, where
144 economic margin per bird is much lower, have included simplified formulations containing
145 fewer parasite species such as Coccivac®-B, Paracox®-5, and Livacox® T, but uptake
146 remains low with only about 5-10% of the poultry produced each year receiving an
147 anticoccidial vaccine. To make a significant impact a third generation of cheaper, and more
148 readily up scalable, anticoccidial vaccines is required.

149

150 **What has happened to the third generation anticoccidial vaccines?**

151 Throughout the 1980s and 1990s, numerous efforts were made to identify parasite antigens
152 and the genes that encode them as novel targets in the development of third generation
153 subunit anticoccidial vaccines (reviewed extensively in [3]). Unfortunately progress has been

154 extremely limited to date, reflecting the general difficulty experienced in the identification of
155 antiprotozoal or antiparasite vaccine candidates and the limitations of testing for
156 immunogenicity, rather than ability to induce immune protection [31]. The likely requirement
157 for more than one antigen if subunit vaccination is to become a viable alternative to
158 chemotherapy has added further complication. Commercial problems including the cost of
159 developing and licensing new vaccines, protecting intellectual property and the risk of
160 resistance developing to any vaccine based on a small number of immunoprotective
161 antigens served to further dampen enthusiasm within the industry. To date only CoxAbic®, a
162 subunit vaccine prepared from *E. maxima* affinity purified gametocyte antigens (APGA), has
163 been successfully commercialized [32, 33]. While CoxAbic® has been reported to be
164 effective in the field [32, 34], in common with the live anticoccidial vaccines it relies on
165 parasite propagation in chickens for its production and remains limited by production
166 capacity and relative expense, further complicated by the need for antigen purification [30].

167

168 Vaccine development strategies have included the testing of recombinant protein, DNA,
169 dendritic cell-derived exosome, and vectored subunit vaccines (Table 1), all relying on the
170 identification of one or more appropriate antigens. Early studies focused on antigens
171 identified on the basis of host immune recognition, and led to the identification of several
172 proteins involved in host-parasite interaction and invasion. Microneme proteins 1 (also
173 identified as Etp100 and TFP100), 2 and 4 (TFP250) were tested as recombinant proteins
174 [35-37]; more recently a rhomboid-like protease linked to invasion has received attention
175 [38], and GPI-linked surface antigens including EtSAG1 (also identified as TA4) have been
176 re-visited and widely tested with mixed results [39-41]. Other immunogenic parasite
177 molecules such as α -tubulin have been tested as recombinant protein [42]. A putative
178 fibronectin was investigated using monoclonal antibodies [43] and various proteins from the
179 sporozoite refractile bodies including SO7 (also identified as RB1 and GX3262), refractile
180 body protein 1A, and lactate dehydrogenase have been tested using multiple approaches
181 [40, 44-47]. Profilin (also identified as 3-1E) has been tested more than any other eimerian
182 antigen as both an anticoccidial vaccine candidate and an adjuvant [48-54]. Attempts have
183 also been made to define the immunoprotective component of the APGA CoxAbic® vaccine
184 through investigations into gametocyte antigens gam56 and gam82 [55-57]. Unfortunately,
185 while partially protective effects have been reported in many of these experimental challenge
186 studies, progress towards vaccine commercialization has not followed for any of them.
187 Efforts to improve efficacy and reproducibility have included production of hybrid proteins,
188 DNA, and synthetic vaccines [40, 58, 59], testing of novel adjuvants including a range of
189 Montanide oil-based adjuvants, ginsenosides and plant saponins [36, 53, 60], and co-

190 administration of avian cytokines including interleukin (IL)-1 β , IL-2, IL-8, IL-15, interferon
191 (IFN) α , IFN γ , transforming growth factor (TGF)- β 4, and lymphotactin [49]. The reasons why
192 none of these approaches have reached field-testing or commercialization remain obscure
193 but clearly indicate lack of reproducible, efficacious vaccine protection. This could be due to
194 inherent insufficiencies in the immunoprotective capacities of the tested antigens or reflect
195 limitations in the vaccine delivery strategies used. Many parasite antigens exposed to the
196 host during *in vivo* replication are highly immunogenic, but the majority of these do not
197 induce immune responses that block re-infection. Ultimately it is highly likely that more than
198 a single antigen will be needed to induce solid immune protection against each *Eimeria*
199 species, suggesting that co-vaccination with multiple existing or new vaccine candidates
200 should be tested as part of future vaccine development programmes.

201

202 **New approaches**

203 Recent changes to legislation governing the use of anticoccidials in many countries such as
204 those within the EU and a general expansion of interest in food security issues have
205 stimulated renewed interest in the development of third generation anticoccidial vaccines.
206 Given the paramount importance of identifying effective vaccine candidates, attention has
207 focused on robust strategies for their identification. Improved understanding of molecules
208 that are essential to host-parasite interaction can highlight new candidates. For example, the
209 use of cell-based and carbohydrate microarray assays to investigate the role played by *E.*
210 *tenella* microneme protein 3 (EtMIC3) in site-specific invasion of the chicken intestine
211 suggested that vaccines based on the microneme adhesive repeat regions (MARR) from this
212 protein may be effective at blocking invasion [61]. Testing single, multiple, and/or hybrid
213 MARR as recombinant protein and DNA vaccines demonstrated significant immune
214 protection against *E. tenella* infection in a series of small-scale challenge studies [61].

215

216 Transcriptome-wide screening approaches have been used recently to identify novel vaccine
217 candidates both *in vivo* and *in silico*. Zhu and colleagues used the eukaryotic expression
218 vector pVAX1.0 to create and test an *E. acervulina* sporozoite expression library [62].
219 Sequential rounds of screening by vaccination identified two candidate cDNA clones (cSZ-
220 JN1 and cSZ-JN2), each of which, when tested individually, induced immune protection in
221 terms of weight gain, lesion score, and oocyst output [62, 63]. While useful in this example
222 the potential for confounding factors should be noted including the risk of combining antigens
223 which induce antagonistic and protective immune responses in the same pool, as described
224 for *Leishmania donovani* [64], as well as the need to choose an immunologically relevant life
225 cycle stage. *In silico* approaches to the same problem rely on access to good quality

226 datasets. Klotz and colleagues generated a cDNA library from *E. tenella* sporozoites and
227 sequenced 191 clones [65]. Working on the hypothesis that many parasite proteins expected
228 to interact with the host immune system are likely to be secreted, all open reading frames
229 predicted to encode a signal peptide sequence were examined, and six were tested for
230 vaccinal potential, highlighting two surface antigen (EtSAG)-like sequences and a putative
231 *Plasmodium falciparum* Pfs40 homologue [65]. The recent expansion in genomic and
232 transcriptomic resources for *Eimeria* species (Reid et al., unpublished) (see
233 <http://www.genedb.org/Homepage/Etenella>) now finally provides resources appropriate for
234 larger, more systematic transcriptome-wide *in silico* analyses.

235

236 Recognizing the importance of immune protection, rather than immune stimulation, also
237 prompted the creation of genome-wide genetic mapping studies to identify vaccine
238 candidates. Building on previous observations that strain-specific immune selection of
239 genetically distinct *E. maxima* strains conferred a highly selectable phenotype [66], genomic
240 regions whose inheritance conferred susceptibility to immune selection were mapped using
241 a 'hitch-hiker' mapping strategy (Figure 2) [67]. Differences in the inheritance of strain-
242 specific genetic markers by progeny of multiple crosses between antigenically distinct *E.*
243 *maxima* strains, with or without strain-specific immune selection, identified six genomic
244 regions likely to encode immunoprotective antigens. Detailed fine mapping of two of these
245 regions has identified apical membrane antigen-1 (EmAMA-1) and immune mapped protein-
246 1 (EmIMP-1) as vaccine candidate antigens. *In vivo* testing of these two candidates using
247 DNA and recombinant protein vaccination has confirmed their annotation as valid
248 anticoccidial vaccine candidates. Amongst the strengths of the genetic mapping approach is
249 the removal of confounding factors such as antigenic dominance, stage-specific protein
250 expression, or site of protein localization, and the fact that immune killing is used as an
251 absolute readout of immunoprotective antigenicity. Again, availability of genomic resources
252 for *Eimeria* species combined with next-generation sequencing technologies promotes
253 extension of genetics-led strategies for vaccine development and improved understanding of
254 other selectable traits.

255

256 Beyond the identification of effective vaccine candidates, the context and environment in
257 which antigens are delivered to hosts are of fundamental importance. DNA and recombinant
258 protein vaccinations have become valuable tools, but the current labour-intensive routes of
259 application suggest that they are unlikely to be accepted as mainstream commercial
260 vaccines for the mass poultry markets. The scale and tight economic margins of poultry
261 production indicate a requirement for low input, easily administered vaccines. For third

262 generation anticoccidial vaccines a number of vectored approaches have been tested with
263 many of the antigens described above. Fowlpox virus (FWPV) and herpes virus of turkeys
264 (HVT) are effective vectors for delivery of *E. acervulina* refractile body transhydrogenase and
265 lactate dehydrogenase under experimental conditions [46]. Both of these viruses are
266 licensed for use in poultry, and a small number of recombinant FWPV and HVT vaccines are
267 commercially available, offering protection against diseases including Newcastle disease,
268 infectious bursal disease, and infectious laryngotracheitis. Several bacterial vectors have
269 also been tested with *Eimeria* antigens under experimental conditions including *Escherichia*
270 *coli* [68], *Salmonella* Typhimurium [69-72], and *Mycobacterium bovis* [73], all of which offer
271 plausible commercial possibilities. Also offering commercial potential are plant vector
272 systems, in which vaccination can theoretically be incorporated into routine dietary
273 components. Preliminary trials in which the microneme proteins EtMIC1 and EtMIC2 have
274 been expressed in the tobacco plant *Nicotiana tabacum* have yielded promising results [74,
275 75]. In other trials expression of single chain variable fragment (scFv) antibodies with affinity
276 for EtSAG1 expressed in seeds of the fodder pea ('Eiffel' variety) reduced *E. tenella*
277 replication in birds following forced feeding [76].

278

279 **Standardising vaccine assessment and development**

280 Over the last 30 years many antigens have been tested as novel anticoccidial vaccine
281 candidates, generating a vast amount of new data on different aspects of parasite biology
282 and identifying potential targets for intervention. Unfortunately lack of standardisation
283 between experiments, research groups, government agencies, and industry has hampered
284 meaningful comparisons. Experimental variables such as the breed/line of chicken and
285 parasite species/strain used [66, 77] as well as considerations including the choice of DNA
286 vaccine vector or recombinant protein expression system, the type of buffer or adjuvant, the
287 dose, frequency and site of immunization, and the specific methods used to measure
288 vaccine efficacy, all exert profound influences on experimental outcomes. Variation in diet
289 has also been shown to significantly influence the results of anticoccidial vaccination using a
290 recombinant protein where phytonutrient concentration affected the type and magnitude of
291 immune response during challenge as well as weight gain and parasite replication [78].
292 While phytotherapy and pre-/probiotics are yet to become established as convincing
293 anticoccidial strategies [79], it is clear that environmental variation has an impact on
294 eimerian replication and is highly likely to influence responses to vaccination. While this
295 problem is not specific to research with *Eimeria*, the establishment of harmonised guidelines
296 for the testing of anticoccidial subunit vaccine candidates, drugs and diagnostics, as has
297 already been described for live vaccines [80], would support best practice and community

298 recognition of the most effective antigens, and could provide a relevant scale of efficacy for
299 new contenders. Ultimately any third generation anticoccidial vaccine will require rigorous
300 testing comparable to that specified by the British and European Pharmacopoeia for live
301 coccidiosis vaccines [81] before it can be registered.

302

303 **New opportunities**

304 Broader biological work that has been ongoing with *Eimeria* parasites is now providing
305 opportunities for improved control of coccidiosis. Protocols supporting genetic
306 complementation and modification by transgenesis are immensely powerful tools for cell
307 biological and genetics studies in several genera of apicomplexan parasites. Recent
308 progress in this field with *Eimeria* allows creation of transgenic parasite lines expressing one
309 or more foreign proteins [82, 83], prompting the notion that *Eimeria* species could be used as
310 host-specific vaccine delivery vectors. Preliminary tests indicated that *E. tenella* is capable of
311 expressing enhanced yellow fluorescent protein (EYFP) as a model antigen that stimulates a
312 range of humoral and cell-mediated immune responses in the chicken following oral
313 vaccination with genetically modified oocysts [84]. More recently, experimental oral
314 vaccination of chickens with *E. tenella* parasites expressing *Campylobacter jejuni* antigen A
315 (CjaA) was found to stimulate an anti-*C. jejuni* immune response and reduce *C. jejuni*
316 colonization of the gut by between 86% and 91% compared to controls [85]. Successful
317 genetic complementation of other *Eimeria* species including *E. acervulina*, *E. maxima*, and
318 *E. praecox*, as well as the rat-specific *Eimeria nieschulzi* [29, 67, 86], support the potential
319 use of these less pathogenic species and strains as vectors to develop novel types of
320 anticoccidial vaccines that may induce immunoprotection against other veterinary or
321 zoonotic pathogens such as *C. jejuni* or avian influenza [87].

322

323 Changing focus to the host, a number of independent genetics studies have compared and
324 quantified *Eimeria* replication in genetically distinct lines of inbred and outbred chicken,
325 revealing between two- and fourfold variations in overall susceptibility to different parasite
326 species [77, 88]. Recent availability of detailed genomic resources for the chicken, including
327 a full draft genome sequence and high-density single nucleotide polymorphism (SNP) arrays
328 [89] now permit the detailed genetic mapping, sequencing, and testing of loci and genes
329 associated with resistance phenotypes.

330

331 **Concluding remarks**

332 Coccidiosis caused by parasitic *Eimeria* species remains one of the greatest burdens on the
333 economics of production of poultry and poultry-derived products. While control is widely

334 available through the use of anticoccidial drugs, and live first and second-generation
335 vaccines, neither of these are completely satisfactory, and new strategies are urgently
336 required. A third generation of more cost-effective anticoccidial vaccines seems to be
337 feasible with several candidate antigens described and many reports of partial protection in
338 experimental settings. However, none of these approaches has progressed into commercial
339 development despite more than 25 years of research [90, 91]. Comparison with progress for
340 other apicomplexans, including high profile human pathogens such as *P. falciparum*, make it
341 clear that whilst vaccines are far from straightforward, they are at least technically possible
342 [92]. Despite many apparently promising candidates falling by the wayside there is now a
343 small panel of realistic vaccine contenders. Future challenges will include identification of
344 optimal delivery strategies and how we respond to parasite evolution in the face of vaccine
345 selection. Of great importance, the genomes of all seven *Eimeria* species that infect the
346 chicken are now sequenced and undergoing analysis and annotation (Reid et al.,
347 unpublished) (see <http://www.genedb.org/Homepage/Etenella>). Such resources will be
348 invaluable in the discovery of novel targets for anticoccidial vaccine or drug intervention and
349 identification of putative homologues in each species.

350

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561

562 **Figure legends**

563 **Figure 1.** A generalised life cycle for parasites within the *Eimeria* genus.

564 1. Ingestion of a sporulated oocyst initiates the endogenous phases of the *Eimeria* life cycle.
565 2. For avian *Eimeria* species the tough oocyst wall is disrupted mechanically during passage
566 through the crop or gizzard, releasing four sporocysts from each sporulated oocyst. For
567 *Eimeria* which infect mammals, enzymatic digestion is likely to be more important as the
568 oocyst traverses the stomach and proximal intestine. 3. Exposure to digestive enzymes
569 allows the sporozoite to escape the sporocyst as it passes through the intestine. The
570 sporozoite continues to pass through the intestinal lumen until it attaches to and invades the
571 epithelial layer. The exact site of invasion varies for each *Eimeria* species [2, 3]. 4. Inside the
572 epithelial cell the sporozoite rounds up into a trophozoite before undergoing schizogony
573 (asexual multiple fission), resulting in the production of multiple first generation merozoites
574 which rupture and leave the host cell. 5. Each first generation merozoite invades another
575 epithelial cell prior to entering a second round of schizogony, leading to production of second
576 generation merozoites. One, two or more rounds of schizogony may follow. 6. After a
577 parasite species-specific finite number of schizogonies the final generation of merozoites
578 differentiate into gametes as the sexual phase of the life cycle begins, forming
579 macrogametocytes, which develop into uninucleate macrogametes (♀), or
580 microgametocytes which produce large numbers of motile, biflagellated microgametes by
581 multiple fission (♂). 7. Mature microgametes leave the host cell and penetrate neighbouring
582 cells, fertilising mature macrogametes to form zygotes. 8. After fertilisation the macrogamete
583 forms a resistant wall as it transforms into an oocyst which escapes from the host cell into
584 the intestinal lumen to be excreted into the environment to initiate the exogenous phase. 9.
585 The unsporulated (non-infectious) oocyst undergoes sporulation in the environment requiring
586 warmth, oxygen and moisture as it undergoes sequential meiotic and mitotic nuclear division
587 to become a sporulated oocyst. The sporulated oocyst, which contains four sporocysts, each
588 of which contain two sporozoites, is now infectious.

589 Each stage of the *Eimeria* life cycle within the host is known to be immunogenic, with
590 the early life cycle stages considered to be most important in the induction of a protective
591 immune response [33, 93, 94]. Few of the current anticoccidial vaccine candidates are
592 expressed throughout the *Eimeria* life cycle and it is likely that multiple antigens will be
593 required if an effective subunit vaccine is to be established.

594

595

596 **Figure 2.** Genetic mapping of loci encoding essential immunoprotective antigens as novel
597 vaccine candidates [67]. **(a)** Antigenically distinct *Eimeria maxima* red and blue strains
598 genotyped at a panel of marker loci (twelve used in this example), two of which are absent
599 from the red strain, three absent from the blue strain. Marker red-6 (highlighted in black) is
600 closely linked to a gene that encodes a strain-specific immunoprotective antigen. **(b)** In the
601 absence of immune selection each hybrid progeny population will contain every marker,
602 which defines either parent within a pool of multiple, genetically heterogeneous clones
603 arising from meiotic segregation and recombination. **(c)** Under red strain-specific immune
604 selection all progeny parasites expressing the red strain-specific antigen will be killed,
605 removing or severely reducing the occurrence of genetically linked marker red-6. Thus,
606 genes closely associated with marker 6 within the red strain genome will be assessed for
607 vaccine candidacy.
608
609

610 **Box 1. Outstanding questions**

- 611 • Which of the 8,000-9,000 antigens encoded within each eimerian genome is capable of
612 stimulating a protective immune response?

613 A small number of defined subunit vaccine candidates have been identified, but none
614 have reached clinical development.

- 615 • If antigens appropriate for use as defined subunit vaccines are identified, how can they
616 be delivered to poultry in a cost effective manner?

- 617 • How can immunisation be optimised in young, immunologically naive chickens?

618 The identification of optimal delivery mechanisms and adjuvants will be crucial.

- 619 • To what extent are *Eimeria* capable of evolving to avoid subunit vaccine-induced
620 immune killing, and how can we minimise this risk?

621

622

623 **Tables**

624

625 **Table 1.** Summary of the most widely tested anticoccidial subunit vaccine candidates.

Vaccine candidate	Form of delivery tested		
	Protein ^a	DNA ^b	Vectored ^c
Apical membrane antigen-1	✓ [67]	✓ [67]	✓ [67]
gam56	✓ [57]	✓ [58]	n/d
gam82	✓ [56]	n/d	n/d
Microneme protein 2	✓ [95]	✓ [96]	✓ [75]
Microneme protein 3	✓ [61]	✓ [61]	n/d
Immune mapped protein-1	✓ [67]	✓ [67]	✓ [67]
Lactate dehydrogenase	✓ [47]	✓ [97]	✓ [46]
Profilin (3-1E)	✓ [78]	✓ [49]	n/d
Rhomboid-like proteins	✓ [98]	✓ [98]	✓ [73]
SO7	✓ [99]	✓ [65]	✓ [69]
TA4	✓ [39]	✓ [58]	✓ [72]

626

627 ^aProtein vaccination: antigen expressed as a recombinant protein (e.g. bacterial expression)
628 prior to vaccination, usually delivered by subcutaneous or other site injection supplemented
629 with an adjuvant such as Freund's Incomplete Adjuvant.

630 ^bDNA vaccination: complete or partial antigen sequence presented within a eukaryotic
631 expression vector under control of a strong promoter designed to drive transcription and
632 translation of the vaccine antigen *in vivo*, usually delivered by intra-muscular or other site
633 injection.

634 ^cVectored vaccination: use of genetically modified virus, bacteria, parasite, yeast or plant to
635 express a vaccinal antigen and deliver it to the vaccinated animal either in a live or killed
636 formulation.

637 Abbreviations: ✓, tested and found to be effective; n/d, not determined.

638

639

640 **Glossary**

641 Anticoccidial chemicals: anticoccidial drugs produced by synthesis, distinct from the
642 ionophores. Examples include decoquinate, diclazuril and robeindine.

643 Antigenic dominance: immunogenic antigens that stimulate a strong immune response and
644 overwhelm other, potentially immunoprotective, antigens. In this example screening immune
645 responses of convalescent animals can reveal the dominant immunogenic, but not
646 necessarily immunoprotective, antigens providing potentially false leads in subunit vaccine
647 development.

648 Attenuated anticoccidial vaccine: live vaccines containing one or more *Eimeria* species
649 parasites attenuated by selection for precocious development or serial passage in
650 embryonated eggs. Attenuation results in reduced reproductive capacity and consequentially
651 a reduced risk of clinical or sub-clinical disease.

652 *Eimeria*: genus of apicomplexan parasite. Seven species are recognized to infect the
653 chicken.

654 Gametocyte: the sexual stages of the *Eimeria* life cycle.

655 Haemorrhagic coccidiosis: disease caused by *Eimeria brunetti*, *Eimeria necatrix* and *Eimeria*
656 *tenella*, characterized by haemorrhagic enteritis.

657 Hitch-hiker genetic mapping: population-based genetic mapping strategy to identify genes
658 which associate with a selectable phenotype through detection of changes in the occurrence
659 of polymorphic, but potentially neutral, genetic markers that are physically linked to a
660 causative locus.

661 Homoxenous faecal-oral life cycle: Single host parasite life cycle, transmitted by ingestion of
662 faecally contaminated environmental material (e.g. food, water, bedding, preening).

663 Ionophores: lipid soluble antimicrobials produced by fermentation. The major drug class
664 used to control *Eimeria*. Examples include monensin, narasin and salinomycin.

665 Malabsorptive coccidiosis: disease caused by *Eimeria acervulina*, *Eimeria maxima*, *Eimeria*
666 *mitis* and *Eimeria praecox*, characterized by mucoid enteritis.

667 Poultry production systems:

668 • Breeder: chicken produced to breed future generations of broiler, layer or other
669 chickens.

670 • Broiler: chicken produced for meat production.

- 671 • Free-range: chickens produced for meat and/or eggs that are allowed freedom to
672 roam for food, usually within an enclosed area but with provision for extensive
673 movement in the open air.
- 674 • Housed: chickens produced for meat and/or eggs enclosed within a building
675 ('house'). House design varies between regions, usually featuring two or more wire
676 walls in tropical and hot regions but enclosed within solid walls in more temperate
677 and colder regions.
- 678 • Layer: chicken produced and maintained for egg production.
- 679 • Organic: chicken production for meat and/or eggs in any form of accommodation
680 achieved without the use of synthetic products, including drugs or growth promoters
681 for the chickens or their food, or genetic modification.

682 Phytotherapy: The use of extracts from natural sources such as plants as medicinal or
683 health-promoting products.

684 Oocyst: a cyst formed by a protozoan parasite. For *Eimeria* this is the environmentally-
685 resistant stage of the life cycle and the infectious unit.

686 Schizogony: asexual reproduction by multiple fission.

687

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