An optimised protocol for molecular identification of *Eimeria* from chickens[☆]

Saroj Kumar[a], Rajat Garg[a,*], Abdalgader Moftah[b], Emily L. Clark[c],
Sarah E. Macdonald[c], Abdul S. Chaudhry[b], Olivier Sparagano[d],
Partha S. Banerjee[a], Krishnendu Kundu[a], Fiona M. Tomley[c], Damer P. Blake[c,**]

[a] *Division of Parasitology, Indian Veterinary Research Institute, Izatnagar 243 122, Uttar Pradesh, India*
[b] *School of Agriculture, Food and Rural Development, Agriculture Building, Newcastle University, Newcastle upon Tyne NE1 7RU, United Kingdom*
[c] *Pathology and Pathogen Biology, Royal Veterinary College, Hawkshead Lane, North Mymms AL9 7TA, United Kingdom*
[d] *Faculty of Health and Life Sciences, Northumbria University, Newcastle upon Tyne NE1 8ST, United Kingdom*

ABSTRACT

Molecular approaches supporting identification of *Eimeria* parasites infecting chickens have been available for more than 20 years, although they have largely failed to replace traditional measures such as microscopy and pathology. Limitations of microscopy-led diagnostics, including a requirement for specialist parasitological expertise and low sample throughput, are yet to be outweighed by the difficulties associated with accessing genomic DNA from environmental *Eimeria* samples. A key step towards the use of *Eimeria* species-specific PCR as a sensitive and reproducible discriminatory tool for use in the field is the production of a standardised protocol that includes sample collection and DNA template preparation, as well as primer selection from the numerous PCR assays now published. Such a protocol will facilitate development of valuable epidemiological datasets which may be easily compared between studies and laboratories. The outcome of an optimisation process undertaken in laboratories in India and the UK is described here, identifying four steps. First, samples were collected into a 2% (w/v) potassium dichromate solution. Second, oocysts were enriched by flotation in saturated saline. Third, genomic DNA was extracted using a QIAamp DNA Stool mini kit protocol including a mechanical homogenisation step. Finally, nested PCR was carried out using previously published primers targeting the internal transcribed spacer region 1 (ITS-1). Alternative methods tested included sample processing in the presence of faecal material, DNA extraction using a traditional phenol/chloroform protocol, the use of SCAR multiplex PCR (one tube and two tube versions) and speciation using the morphometric tool COCCIMORPH for the first time with field samples.

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1. Introduction

Coccidiosis, caused by protozoan parasites belonging to the genus *Eimeria*, is one of the commonest and most economically important enteric diseases of chickens' worldwide (Shirley et al., 2005). Seven *Eimeria* species can infect the chicken (viz., *Eimeria acervulina*, *Eimeria brunetti*, *Eimeria maxima*, *Eimeria mitis*, *Eimeria necatrix*, *Eimeria praecox* and *Eimeria tenella*) and all can

* Corresponding author.
** Corresponding author. Tel.: +44 1707 666041.
E-mail addresses: rajatgarg_2000@yahoo.com (R. Garg),
dblake@rvc.ac.uk (D.P. Blake).

compromise economic production and animal welfare, resulting in poor feed conversion ratios, failure to thrive and elevated mortality (Long et al., 1976; Williams et al., 2009). Conventionally, identification of *Eimeria* spp. is based on morphological features of the sporulated oocyst, sporulation time and location/scoring of pathological lesions in the intestine but the procedures involved require specialist expertise and have serious limitations due to their subjective nature and overlapping characteristics among different species (Long and Joyner, 1984). Mixed infections also pose a problem for the precise discrimination of species using morphological methods. Alternative species-specific diagnostics are required to inform routine animal husbandry, veterinary intervention and epidemiological investigation.

One such alternative is *Eimeria* species-specific polymerase chain reaction (PCR). Over the last 20 years several PCR assays have been developed that target genomic regions of one or more *Eimeria* species including the *E. tenella* 5S or small subunit rRNAs (Stucki et al., 1993; Tsuji et al., 1999), the first and second internal transcribed spacer regions (ITS-1 and -2) (Gasser et al., 2001; Lew et al., 2003; Schnitzler et al., 1998; Su et al., 2003; Woods et al., 2000) and gene-specific targets including sporozoite antigen gene EASZ240/160 (Molloy et al., 1998). In one of the most comprehensive studies Fernandez et al. (2003) designed species-specific primers for *Eimeria* spp. from a group of SCAR (Sequence-Characterized Amplified Region) markers and used them to develop a multiplex PCR for the simultaneous discrimination of different *Eimeria* spp. in a single reaction. Importantly, many of these assays have been shown to be capable of detecting genomic DNA representing as few as 0.4–8 oocyst-equivalents (Fernandez et al., 2003; Haug et al., 2007), or as few as 10–20 oocysts (Carvalho et al., 2011a; Frölich et al., 2013). Nonetheless, routine application with field samples remains complicated by factors including DNA extraction from within the tough oocyst wall and faecal PCR inhibition (Raj et al., 2013). Broader uptake of PCR-based *Eimeria* diagnostics can be significantly enhanced by establishment of an optimised protocol. Similarly, identification of the most sensitive and robust primers from the large number of *Eimeria*-specific PCR assays that are available is an essential step towards standardised epidemiological analyses appropriate for international comparison. Validation of collection, purification and PCR amplification protocols across different labs, in multiple countries, is a key step in the establishment of optimal sampling strategies as we seek to improve understanding of parasite field biology.

Beyond PCR other approaches to species-specific identification of *Eimeria* include quantitative PCR (qPCR) (Morgan et al., 2009; Vrba et al., 2010), although cost is currently limiting for routine applications, and Loop-mediated Isothermal Amplification (LAMP; Barkway et al., 2011). Importantly, accessing DNA from within the robust oocyst wall is a challenge for all of these technologies when working with faecal or litter samples. An alternative computational approach is the use of software tool COC-CIMORPH (http://www.coccidia.icb.usp.br/coccimorph), which is based on identification of sporulated oocysts of *Eimeria* spp. of poultry by morphological analysis (Castañón et al., 2007).

In the present study three different parasite purification/DNA extraction procedures (QIAamp Stool Mini kit with and without faecal contamination, and phenol/chloroform) and three different PCR protocols (nested PCR ITS-1 amplification and multiplex SCAR PCR in a one or two tube format) have been tested in India and the UK and compared to the software tool COCCIMORPH for diagnostic efficacy on coccidia positive faecal droppings collected from commercially raised poultry.

2. Materials and methods

2.1. Faecal sample collection

During November 2011 to April, 2012, a total of 45 commercial poultry farms were sampled from Uttar Pradesh and Uttarakhand states of North India. During the same period 139 commercial poultry farms in Egypt, Libya and the UK were sampled. For collection of poultry droppings 50 ml polypropylene conical tubes were used, each with a screw top and containing 5 ml potassium dichromate (2% w/v). The weight of each tube was recorded and pooled faecal droppings were collected starting from one corner of a unit and following a 'W' pathway across the unit, collecting one fresh dropping every two to five paces depending on the size of the unit until the tube was filled to the 10 ml mark. Three to five tubes were filled per unit. Each tube was then properly capped and the contents were thoroughly mixed by vigorous shaking. The samples thus collected were transported to the laboratory and refrigerated at 4 °C until further processed.

2.2. Processing of faecal samples

The tubes with faecal material were again weighed and 1.6 g sodium chloride was added to each tube. Then saturated salt solution was added up to the 25 ml mark. The tubes were capped tightly and vigorously shaken until the faecal material was completely broken and mixed well. Finally, the tubes were filled up to 50 ml mark with saturated salt solution and mixed thoroughly. On this faecal suspension, 1–2 ml of single distilled water was gently overlaid. The sample was left to stand for ten minutes and then centrifuged at ∼750 × g for 8 min. Using a disposable Pasteur pipette, the layer from the interface between the saturated salt and the water was transferred to a new 50 ml polypropylene conical tube. This was continued for three more times till no material was visible at the interface. The new tube was filled up to 50 ml mark with single distilled water and centrifuged at ∼750 × g for 8–10 min. The supernatant was carefully removed without disturbing the pellet using a disposable Pasteur pipette, leaving 3–5 ml fluid. The supernatant was checked microscopically for unpelleted oocysts before discarding.

The sample from the above step was transferred into a 2.0 ml microfuge tube, taking care to mix the sample and rinse the sides up to ∼3 cm from the base of the 50 ml tube. The microfuge tube was then centrifuged at ∼6000 × g for 5 min and the supernatant was discarded after microscopic

screening for unpelleted oocysts. The pelleted oocysts were suspended in 1.0 ml distilled or molecular grade water. After through mixing, 10 μl of this sample was drawn from the microfuge tube and mixed with saturated salt solution up to the 1 ml mark for estimating the final oocyst concentration (oocysts per gram of faeces, OPG) in the sample using McMaster chambers. The eimerian oocysts were then allowed to sporulate in 2% w/v potassium dichromate solution at $27 \pm 2\,°C$ for three days. Following sporulation, the oocysts were thoroughly washed thrice in autoclaved distilled or molecular grade water for taking photomicrographs and pelleted for DNA isolation.

2.3. Identification of Eimeria spp. by COCCIMORPH

For the identification of eimerian oocysts, photomicrographs of at least 50 individual sporulated oocysts were randomly taken from each sample at $10\times/40\times$ using a dry high power objective with a photomicrographic camera (Moticam5, Hong Kong) attached to a trinocular research microscope (Motic Trinocular Research Microscope BA210, Hong Kong). The identification of *Eimeria* spp. of chickens was done using COCCIMORPH software (http://www.coccidia.icb.usp.br/coccimorph/). The software was downloaded from the Internet and the oocyst images (400× magnification) were uploaded for species identification as described online. The *Eimeria* spp. identified by the software in each sample was recorded.

2.4. Isolation of genomic DNA

For isolation of genomic DNA, only samples found to contain more than 500 (India) or 200 (Egypt, Libya and UK) OPG were selected for processing.

2.4.1. QIAamp DNA Stool mini kit

Total genomic DNA was isolated using a QIAamp DNA Stool mini kit (Qiagen, Germany) as per the manufacturer's protocol with some modifications from (i) oocysts purified as described above or (ii) purified oocysts supplemented with 100 mg oocyst-negative faecal material collected from a specific pathogen free chicken to mimic the absence of a flotation step. Briefly, to the pelleted oocysts an equal volume of autoclaved glass ballotini beads measuring ~0.25–0.5 mm in diameter (Sigma–Aldrich, USA) were added and covered with a minimum volume ASL buffer (out of total 1.4 ml to be used for DNA isolation) supplied with the DNA extraction kit or sterile TE buffer. The oocysts were then disrupted by vortexing (India; Spinix Vortex Shaker, Tarsons, India; maximum speed) or bead-beating (Egypt, Libya and UK, Mini Beadbeater-8, Biospec Products, Bartlesville, USA; set to homogenise) for two minutes. Then, the remaining buffer ASL was added to the tube and thoroughly mixed. The suspension was then heated for 5 min at 70 °C and processed as per the QIAamp DNA Stool kit protocol. The DNA was eluted twice in 100 μl TE buffer as recommended by the manufacturer and quantified using absorbance at 260 and 280 nm.

2.4.2. Phenol/chloroform DNA extraction

Total genomic DNA was isolated from purified oocysts using a standard phenol/chloroform extraction protocol following disruption using a Mini Beadbeater-8 as described previously (Blake et al., 2003).

2.5. PCR amplification

A summary of the PCR assays tested, and the primers used, is provided in Supplementary Table 1.

2.5.1. Identification of Eimeria genus genomic DNA by PCR

The presence of *Eimeria* genus genomic DNA was tested by PCR amplification of the partial 18S rDNA sequence using the primers ERIB1 and ERIB10 as described elsewhere (Schwarz et al., 2009). Briefly, each reaction contained 2 μl genomic DNA template, 25 pmol forward and reverse primer, 0.5 U *Taq* polymerase (Invitrogen, Paisley, UK), 10 mM Tris–HCl, 1.5 mM MgCl₂, 50 mM KCl and 200 μM dNTPs. Standard cycle parameters were $1\times$ (5 min at 94 °C), $30\times$ (30 s at 94 °C, 30 s at 57 °C, 2 min at 72 °C) and $1\times$ (10 min at 72 °C). Post-amplification PCR products were resolved by agarose gel electrophoresis.

2.5.2. Identification of Eimeria spp. by nested PCR

The nested PCR protocol using ITS-1 primers was standardised for identification of *Eimeria* species of poultry. Primers amplifying the entire ITS-1 sequence with flanking partial 18S rDNA and 5.8S rDNA regions of *Eimeria* were used in the genus-specific PCR phase, while species-specific primers targeting the ITS-1 region were used to amplify the individual *Eimeria* species as described elsewhere (Lew et al., 2003).

Briefly, each 25.0 μl PCR reaction included 2 μl of genomic DNA, 25 pmol each of genus-specific primers, 1.25 U of *Taq* polymerase, 200 μM each of dNTPs, and 2.5 μl of PCR buffer containing 1.5 mM MgCl₂. The thermal cycling was done with an initial denaturing step at 94 °C for 3 min followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 90 s and a final extension at 72 °C for 7 min. The product of the primary PCR (1.0 μl in 25.0 μl reaction mixture) was used as template for the nested PCR with species-specific primers in individual tubes using the same amplification conditions described above excepting different annealing temperatures for different *Eimeria* spp. (58 °C for *E. mitis*; 61 °C for *E. necatrix* and *E. praecox*; 65 °C for *E. tenella*; 71 °C for *E. acervulina*, *E. maxima* and *E. brunetti*). Negative, no-template controls were included with each assay using triple distilled water in place of template. The amplification of specific nested PCR product was checked by gel electrophoresis in 2% agarose gels stained with 0.5 μg/ml ethidium bromide.

2.6. Identification of Eimeria spp. by multiplex PCR

The multiplex PCR using SCAR primers for identification of the seven *Eimeria* species that infect chickens (Fernandez et al., 2003) was standardised using pure DNA samples from the Houghton strains of each *Eimeria* spp.

Initially, the PCR amplification was standardised separately for each species using specific primer pairs (0.55 μM for *E. tenella*, *E. maxima* and *E. mitis*; 0.7 μM for *E. acervulina*, *E. necatrix* and *E. praecox*; 0.85 μM for *E. brunetti*), 200 μM

dNTP, 5.0 mM MgCl$_2$, 3.5 U *Taq* DNA Polymerase, and 1.6×
amplification buffer (supplied by the manufacturer) in a
final volume of 25 μl reaction mixture. Thermocycling con-
ditions were set at 96 °C for 5 min for initial denaturation,
followed by 30 cycles of 1 min at 94 °C, 2 min at 65 °C
and 90 s at 72 °C, with a final extension at 72 °C for 7 min.
Once the above conditions were standardised for individ-
ual primer pairs, all the primer pairs were put together in a
single 50 μl reaction mixture for single-tube multiplex PCR
with the same cycling conditions as described above. For
two-tube multiplex PCR, amplifications were conducted
separately in two tubes; tube 1 contained the primers for
E. acervulina, *E. brunetti* and *E. mitis* while tube 2 contained
primers for *E. maxima*, *E. necatrix*, *E. praecox* and *E. tenella*.
All the conditions for PCR remained as described above. The
amplification of specific PCR products were checked by gel
electrophoresis in 2% agarose gels stained with 0.5 μg/ml
ethidium bromide.

2.7. Statistical analysis

The results of *Eimeria* species detection for each assay
were compared by Chi-square analysis using SPSS version
20 (IBM, US). Results were considered significant when
$p < 0.05$.

3. Results

3.1. Genomic DNA extraction

3.1.1. Protocol selection
Triplicate environmental faecal samples were collected
from 30 farms and examined microscopically to confirm
the presence of *Eimeria* oocysts (10×/20×). Oocysts were
purified, pooled per farm to standardise and split for par-
allel processing by (i) QIAamp DNA Stool kit, (ii) QIAamp
DNA Stool kit plus faecal contamination and (iii) phe-
nol/chloroform. Using the *Eimeria* genus 18S rDNA assay
93% (28/30) of the samples processed using the QIAamp
DNA Stool kit were PCR positive and 100% of the samples
containing ≥5000 OPG at the beginning of the process were
positive (Table 1). The addition of faecal material reduced
the PCR positive rate to 30% with only one of 17 samples
containing fewer than 20,000 oocysts found to be positive.
Using phenol/chloroform extraction 77% (23/30) samples
were PCR positive with a 100% success rate only occurring
above 20,000 starting OPG.

3.1.2. Protocol sensitivity
The protocol found to be most effective (QIAamp DNA
Stool kit after oocyst flotation) was subsequently tested on
a larger number of field samples to investigate diagnos-
tic sensitivity. In total 139 farms were visited, of which
100 were positive (71.9%) for *Eimeria* oocysts by micro-
scopic examination with OPG ranging from 0.2×10^3 to
191.3×10^3. All oocyst positive samples were processed.
Using the *Eimeria* genus 18S rDNA assay 96% (96/100)
of the samples were PCR positive and 100% of samples
containing ≥5000 OPG were positive (Table 2). Sensitivity
dropped below 80% only when samples containing fewer

than 500 OPG were processed, although the number of
samples tested at this level was very small.

3.2. Optimal identification of Eimeria spp.

Out of 45 poultry farms screened in North India, 37
(82.2%) were positive for *Eimeria* spp. by microscopic exam-
ination with OPG ranging from 0.1×10^3 to 242.5×10^3. Out
of the 37 coccidia positive farms, 30 farms had OPG lev-
els above 500 and thus were selected for further *Eimeria*
species identification studies.

3.2.1. COCCIMORPH
COCCIMORPH is a computational approach for parasite
identification in case of *Eimeria* spp. from the chicken. Digi-
tal images of 50 individual unidentified sporulated oocysts
of *Eimeria* spp. were uploaded on to the software. The
software then analysed the oocyst on the basis of dif-
ferent features namely, curvature characterisation, size,
symmetry and internal structure characterisation for the
identification of eimerian species. Identification of *Eimeria*
spp. using COCCIMORPH software revealed the presence
of *E. acervulina*, *E. maxima*, *E. mitis*, *E. praecox*, *E. necatrix*
and *E. tenella*, in 96.7%, 36.7%, 90.0%, 3.3%, 23.3% and 16.7%
of farms, respectively (Fig. 1, Supplementary Table 2). *E.
brunetti* was not recorded in any of the farms screened
using COCCIMORPH.

3.2.2. Nested ITS-1 PCR
Nested PCR using ITS-1 primer was standardised with
pure DNA of all seven species of *Eimeria*. Specific PCR ampli-
cons of *E. acervulina* (321 bp), *E. brunetti* (311 bp), *E. maxima*
US strain (145 bp), *E. maxima* Australian strain (145 bp),
*E. mitis*1 (328 bp), *E. mitis*5 (193 bp), *E. necatrix* (383 bp),
E. praecox (116 bp) and *E. tenella* (278 bp) were visualised
(data not shown). In field samples, ITS-1 based nested PCR
identified *E. acervulina*, *E. brunetti*, *E. maxima*, *E. mitis*, *E.
praecox*, *E. necatrix* and *E. tenella* in 93.3%, 10.0%, 86.7%,
96.7%, 66.7%, 80.0% and 100% farms, respectively (Fig. 1,
Supplementary Table 2). In 16 farms, both the Australian-
and US-type strains of *E. maxima* were identified, while in
ten farms only the US-type strain of *E. maxima* was present.
Similarly, *E. mitis* was identified by primers specific for both
*E. mitis*1 and *E. mitis*5 in all the farms that were positive
for *E. mitis.* Mixed infections of *Eimeria* spp. were recorded
in all farms with a minimum of at least three species (in
four broiler farms). All seven *Eimeria* spp. were identified
in three farms.

3.2.3. SCAR multiplex PCR
Multiplex PCR using SCAR primers was standardised
with pure DNA of all seven species of *Eimeria*. Amplicons
of *E. acervulina* (811 bp), *E. brunetti* (626 bp), *E. maxima*
(272 bp), *E. mitis* (460 bp), *E. necatrix* (200 bp), *E. praecox*
(354 bp) and *E. tenella* (539 bp) were visualised with indi-
vidual primer pairs as well as in multiplex PCR (data not
shown). In field samples, the one-tube multiplex PCR could
identify *E. maxima*, *E. mitis*, *E. necatrix*, *E. praecox* and *E.
tenella*, in 16.7%, 3.3%, 43.3%, 3.3% and 13.3% farms, respec-
tively. *E. acervulina* and *E. brunetti* were not identified in any

Table 1

Comparison of three DNA extraction protocols for the detection of eimerian genomic DNA within chicken faecal samples by PCR targeting the *Eimeria* genus 18S rDNA.

OPG	*n*	Stool kit +F	Stool kit −F	Phenol/chloroform −F
<1000	6	0	5	3
1000–5000	5	1	4	3
5001–20,000	6	0	6	4
20,001–100,000	7	3	7	7
100,001–200,000	6	5	6	6
Total	30	9	28	23

OPG, oocysts per gram starting material; Stool kit, QIAamp DNA Stool kit. +F, including contaminating faecal material; −F, without contaminating faecal material; *n*, number samples tested per OPG group.

Table 2

The influence of faecal sample oocyst concentration on PCR sensitivity for eimerian genomic DNA. Samples prepared using the optimal oocyst flotation/QIAamp DNA Stool kit DNA extraction protocol with a PCR targeting the *Eimeria* genus 18S rDNA.

OPG	Number farms	Theoretical oocysts per PCR[a]	Number positive	Percent positive
<500	5	<25	3	60
500–1000	5	25–50	4	80
1001–2000	5	50–100	5	100
2001–5000	10	100–250	9	90
5001–10,000	20	250–500	20	100
10,001–50,000	36	500–2500	36	100
50,001–100,000	15	2500–5000	15	100
100,001–200,000	4	5000–10,000	4	100
Total	100		96	

OPG, oocysts per gram starting material.

[a] Theoretical oocysts per PCR calculated based upon processing 5 g faeces with DNA elution during extraction in 200 μl and inclusion of 2 μl per PCR.
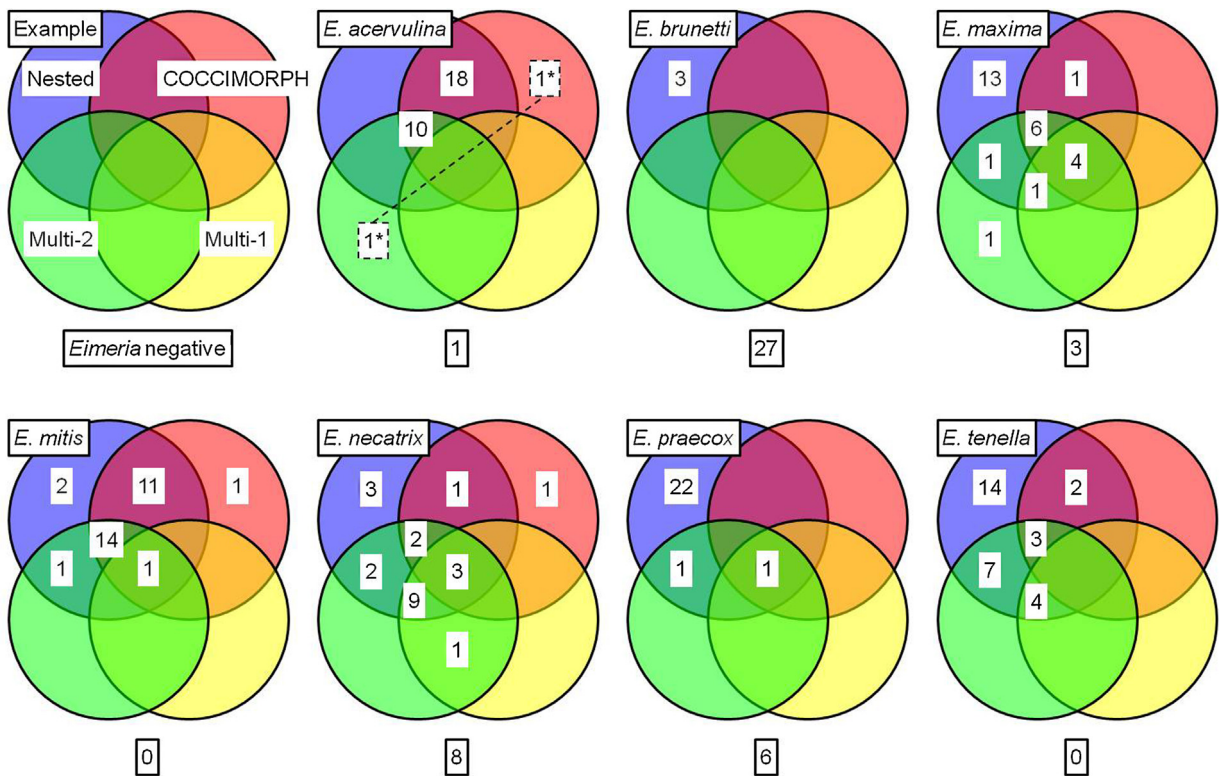
Fig. 1. Summary of *Eimeria* species identification from faecal samples collected on 30 farms in North India. Key as shown in the first panel (Example): blue = identification by nested ITS-1 PCR, red = COCCIMORPH, yellow = SCAR multiplex (one-tube format), green = SCAR multiplex (two-tube format), negative (box external to the Venn diagram) = the number of samples not found to contain *Eimeria*. Data presented in full in Supplementary Table 2. *Denotes a single *E. acervulina* result identified by COCCIMORPH and two-tube SCAR multiplex but not nested ITS-1 or one-tube SCAR multiplex as indicated by a joining broken line. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

of the farms screened by one-tube multiplex PCR. A maximum of two *Eimeria* spp. were identified in six farms, while for 11 farms no *Eimeria* spp. were recorded by one-tube multiplex PCR. However, two-tube multiplex PCR identified *E. acervulina*, *E. maxima*, *E. mitis*, *E. praecox*, *E. necatrix* and *E. tenella*, in 36.7%, 43.3%, 53.3%, 56.7%, 6.7% and 46.7% farms, respectively (Fig. 1, Supplementary Table 2). A maximum of five *Eimeria* species were identified in five farms, while in two farms no *Eimeria* spp. were detected by two-tube multiplex PCR. *E. brunetti* was never identified using the multiplex PCR in one- or two-tube formats.

4. Discussion

Accurate identification of *Eimeria* spp. is important not only for the diagnosis of disease but also for management of subclinical infection, development and application of effective control strategies, and biological and epidemiological study (Lee et al., 2010; Sun et al., 2009). Traditionally, identification of *Eimeria* spp. has been based on the morphological characteristics of oocysts, parasite biology, clinical signs of the affected animals, and the typical macroscopic lesions assessed during necropsy (Long and Joyner, 1984). However, in a natural setting mixed infections of different *Eimeria* spp. are commonly encountered and morphological characteristics and pathological changes may overlap, hindering accurate diagnosis and undermining detection of subclinical disease (Long and Joyner, 1984; Rice and Reid, 1973). Thus, it has been suggested that these methods should not be used in isolation for differentiation of *Eimeria* species (Long and Joyner, 1984; Lopez et al., 2007). Alternatives include molecular or computational approaches such as PCR, qPCR and the software COCCIMORPH. PCR assays capable of identifying and differentiating *Eimeria* spp. have been available for more than 20 years but, despite recognition as the 'gold standard' of detection for many pathogens, this technology is yet to replace traditional coccidial diagnostics (Brook et al., 2008; Olano and Walker, 2011; Stucki et al., 1993). Features of eimerian biology including the resistance of the oocyst wall to anything other than mechanical disruption, limiting access to template DNA (for most avian-infecting species), and PCR inhibition by the surrounding faecal material have discouraged use of PCR. While several PCR assays have been described to identify specific *Eimeria* species very few studies have focused on the applicability of these techniques for identifying *Eimeria* spp. in commercially raised poultry throughout the world (Carvalho et al., 2011a,b; Frölich et al., 2013; Haug et al., 2008). Development of a standardised protocol supporting medium throughput diagnostic sampling for *Eimeria* will enhance the value of such data while promoting the application of PCR and comparison between studies.

Following collection of fresh environmental faecal samples we explored two DNA extraction procedures and the influence of residual faecal contamination. The inclusion of faecal material dramatically reduced PCR sensitivity with genomic DNA purified using the QIAamp DNA Stool kit, supporting the value of even a rudimentary pre-extraction parasite purification step. The cause of this inhibition remains unclear at present. The InhibitEx step of the Stool kit protocol is designed to adsorb substances that can degrade DNA and inhibit downstream enzymatic reactions and should minimise PCR inhibition. While it is possible that the faecal PCR inhibitor concentration over loaded the InhibitEx matrix it is more likely that the residual faecal debris reduced the efficiency of the column purification step. In support of this hypothesis comparable studies using sieved faecal samples with and without flotation were not similarly affected, although this protocol was not adopted owing to quality control issues avoiding contamination between samples during processing (data not shown). Using *Eimeria* oocysts enriched by flotation in saturated saline considerably improved PCR sensitivity, where the Stool kit performed considerably better than the phenol/chloroform extraction (93% compared to 77%). Extension of these studies to include a larger sample panel with the Stool kit revealed an overall sensitivity of 96%, with 100% accuracy when starting with an OPG in excess of 5000 (the equivalent of 250 oocysts per PCR from the beginning of the protocol). DNA precipitation could be considered to concentrate the DNA template and improve PCR sensitivity, although the additional complexity is likely to be limiting in a medium throughput surveillance system. Thus, the low false negative rate and the improved health and safety associated with a non-phenol based protocol supported adoption of the parasite flotation/QIAamp DNA Stool kit protocol.

A comparison of the two most widely studied PCR assays for identifying the *Eimeria* spp. of poultry in field samples (viz., multiplex PCR based on SCAR markers and nested PCR based on amplification of ITS-1 region of the parasite) was also made in the present study. Multiplex PCR based on SCAR amplification for the simultaneous identification of *Eimeria* spp. of the chicken was first described 10 years ago (Fernandez et al., 2003). While the assay performed well with purified genomic DNA its sensitivity and breadth of species identification was reduced when applied to the field samples in common with previous reports (Frölich et al., 2013). Diagnostic multiplex PCR systems used for primary detection of infectious agents are difficult to optimise and suffer from inherent disadvantages of low sensitivity and reproducibility, hindering comparison between laboratories. Additionally, the performance of multiplex PCR is directly dependent upon the final concentration of PCR inhibitors and the concentration of DNA of individual infectious agents in the DNA template (Haug et al., 2007). Better results achieved when dividing the multiplex into two tubes in the present study is notable, offering a compromise between sensitivity and utility in agreement with Carvalho et al. (2011a). Chi-square analysis of the results obtained from the field samples using each technique identified significant differences between all assays ($p < 0.05$), illustrating the importance of selecting and retaining a single, standardised procedure if comparable results are to be generated. Application of the ITS-1 nested PCR assay described previously by Lew et al. (2003) identified more *Eimeria* spp. from more farms, benefiting from a multi-copy genomic target and a nested PCR strategy. The requirement for two PCR steps adds complexity, time and expense to the nested assay but the improved sensitivity was distinct.

Molecular identification of *Eimeria* spp. using PCR was supplemented during these studies by the online COC-CIMORPH tool, an innovative approach developed for identification of eimerian oocysts of poultry and rabbits in which digital images of unidentified sporulated eimerian oocysts are uploaded for species identification on the basis of sporulated oocyst morphology (Castañón et al., 2007). COCCIMORPH was most effective with *E. acervulina* and *E. mitis*, demonstrating good agreement with the nested ITS-1 PCR assay, although it fared less well with *E. brunetti*, *E. praecox* and *E. tenella*. Indeed *E. brunetti* was not identified in any sample, although the occurrence of this species was found to be low throughout the study. Perusal of available literature revealed that no data exists on the use of this software for identification of *Eimeria* spp. in field samples. It has long been recognised that the size and shape ranges of eimerian oocysts are wide, overlap substantially between species (Long et al., 1976) and may vary due to environmental and physical factors (Jones, 1932; Joyner, 1982). Further, infrequent species can remain undetected using COCCIMORPH given that a small subsample may not present a true representation of the total sample. As such, while COCCIMORPH can be a valuable tool for preliminary screening/identification purposes or in the absence of a laboratory it should be reinforced with microscopic or molecular validation.

Comparison of the identification technologies tested here promote use of the nested ITS-1 PCR assay as it was able to identify all of the *Eimeria* spp. that were identified by SCAR multiplex PCR and/or COCCIMORPH with just four exceptions (one *E. acervulina*, one *E. maxima* and two *E. necatrix*; Fig. 1 and Supplementary Table 2). These gaps may have been due to variations in the ITS-1 sequence, as has been reported previously in the case of *E. tenella* from India (Bhaskaran et al., 2010). While it is clear that PCR can facilitate the detection of minority *Eimeria* species subpopulations which may be missed by routine microscopy (Frölich et al., 2013), the reliance of PCR on very small primer annealing sites within a target genome also risks false negatives where genetic diversity occurs. Relevant ITS-1 diversity has already been described for *E. maxima* and *E. mitis*, reflected by the inclusion of multiple primer pairs in the nested PCR (Lew et al., 2003; Schnitzler et al., 1999). Indeed it should be noted from the present study that both the US and Australian ITS-1 *E. maxima* sequence types were evident in North Indian poultry. Thus, while the nested ITS-1 assay provided the best species coverage with a low false negative rate, additional assays will be important if comprehensive surveillance is required.

Identification of chicken *Eimeria* species is of utmost importance for effective control of clinical and subclinical coccidiosis. Conventional parasitological techniques are time consuming and require expertise, which is increasingly expensive and scarce. Computational identification on the basis of oocyst morphology (COCCIMORPH) provides a valuable diagnostic tool but failed to correctly identify many species in practical field application. The use of molecular biological techniques to discriminate between different species of poultry coccidia has been limited to date but the provision of protocols supporting their cost-effective, robust and straightforward application with an easy to interpret output can improve uptake in developed and developing regions. As the cost of PCR equipment and reagents continues to drop, it is feasible that the protocols described here will be developed and integrated into routine poultry management and veterinary surveillance.

Acknowledgements

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetpar.2013.09.026.

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