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Development and validation of real-time PCR assays specific to four species of *Eimeria*

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Short title: *Eimeria* species-specific real-time PCR

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Abstract

The development of quantitative real-time PCR assays specific to *Eimeria acervulina*, *Eimeria maxima*, *Eimeria necatrix* and *Eimeria tenella* is described and validated. PCR templates adopted include a fragment of a gene encoding a microneme protein and previously characterised species-specific RAPD sequences. The sensitivity of each assay allowed the consistent detection of between one and ten parasite genomes, equivalent to between one or two sporulated oocysts or a fraction of a single mature schizont, unaffected by the presence of chicken (host) or other *Eimeria* species DNA. Regression coefficients in excess of 0.99 over linear ranges of at least six orders of magnitude, together with comparable PCR efficiencies, demonstrated the robust reproducibility of each assay and suggest that two or more may be successfully multiplexed. The species-specific assays described here, combined with a previously published generic *Eimeria* species real-time PCR, provide valuable components in a 'tool box' to accurately quantify the presence of specific *Eimeria* species in environmental or within-host phases of the lifecycle with little specialist knowledge. The application of these assays may benefit chicken husbandry, veterinary practice, quality control of live vaccine production and scientific research.

Introduction

The *Eimeria* species are obligate intracellular protozoan parasites that exhibit a monoxenous, self-limiting lifecycle. Infection with *Eimeria* species can result in the disease coccidiosis. *Eimeria* can parasitise all domestically reared livestock in a host-specific manner but, economically, the seven species that infect the chicken may be considered the most important, incurring annual costs predicted to be as high as £1500 million (~US\$3000 million) per annum (Shirley *et al.* 2005). Among these seven species *Eimeria acervulina*, *Eimeria maxima*, *Eimeria necatrix* and *Eimeria tenella* are frequently considered to pose the greatest threat to chicken production, largely due to a combination of pathogenicity and distribution (McDougald *et al.* 1997; Chapman *et al.* 2002). Whilst the specific strain of each parasite may vary in different regions of the world, these four species consistently compromise economic animal production and welfare (McDougald *et al.* 1997; Al-Natour *et al.* 2002; Ashenafi *et al.* 2004).

Eimerian parasites exhibit a faecal/oral transmission mechanism. The environmental phase of the lifecycle, the oocyst, is characteristically tough and provides straightforward access for enumeration and isolation. Indeed, oocyst enumeration has been widely employed to assess degree of infection (Shirley and Hoyle 1981; Bumstead *et al.* 1995; Rose *et al.* 2000; Smith *et al.* 2002).

Unfortunately, identification of species based upon characteristics such as oocyst morphology and pre-patent period can be difficult, even for an expert. Quantification of different species in a sample derived from a mixed infection (frequently found in samples collected from the field) can be costly and extremely time-consuming.

Species-specific quantification of *Eimeria* replication within the host has proven to be similarly problematic, based upon labour-intensive microscopy, informed by site of

infection (an overlapping trait for many of the *Eimeria* species; Long *et al.* 1976). The development of real-time PCR assays targeting *E. acervulina*, *E. maxima* and the generic *Eimeria* species have begun to provide the tools necessary to accurately quantify eimerian infection (Blake *et al.* 2006; Swinkels *et al.* 2006). However, a prerequisite for the application of any such assay in the field, or in a mixed infection model, is validation of target specificity and sensitivity. We report here the development of three new real-time PCR assays and their validation, together with a fourth previously published assay (Blake *et al.* 2006), as specific to the four most economically important *Eimeria* species that infect the chicken.

Materials and Methods

Parasites. The Houghton (H) strains of *E. acervulina*, *E. maxima* and *E. tenella* were used throughout these trials, all of which were the progeny of single oocysts isolated at the Houghton Laboratory (formerly the Houghton Poultry Research Station) of the Institute for Animal Health (UK). The *E. necatrix* GD strain used was isolated in Guangzhou at the Guangdong Academy of Agricultural Sciences (China) and was also derived from infection with a single oocyst. These parasites are passaged at frequent intervals through dosing and recovery as described previously (Long *et al.* 1976).

Animals. The *Eimeria* species parasites used in these trials were routinely passaged and amplified in Light Sussex chickens maintained under specific pathogen-free conditions at the Institute for Animal Health (UK; *E. acervulina*, *E. maxima* and *E.*

tenella) or Huiyang chickens at the Guangdong Academy of Agricultural Sciences (China; *E. necatrix*).

Genomic DNA preparation. Genomic DNA was extracted from purified *E. acervulina*, *E. maxima*, *E. necatrix* and *E. tenella* oocysts as described previously following a BeadBeater and standard phenol/chloroform extraction protocol (Blake *et al.* 2003). The genomic concentration of each extracted sample was calculated using a validated generic *Eimeria* species real-time PCR (Blake *et al.* 2006) and confirmed by gel electrophoretic comparison with standards of known DNA concentration (based upon a predicted genome size of 55 Mb; Sambrook and Russell, 2001). Chicken genomic DNA was prepared as described previously (Blake *et al.* 2006). Aliquots of *Eimeria brunetti*, *Eimeria mitis* and *Eimeria praecox* genomic DNA were generously provided by F. Tomley and K. Billington (Institute for Animal Health, UK).

Primer design. All primers described in this paper were designed using Primer3 software (Rozen and Skaletsky 2000) and manufactured by Sigma-Genosys Ltd. (Haverhill, UK; Table 1). Real-time PCR hybridisation probes were manufactured by Eurogentec Ltd. (Southampton, UK), incorporating a 6-carboxyfluorescein (FAM) fluorophore at the 5' end and a 6-carboxy-tetramethyl-rhodamine (TAMRA) quencher at the 3' end (Table 1).

Polymerase chain reaction amplification of real-time PCR targets. Primers complimentary to species-specific *E. acervulina*, *E. necatrix* and *E. tenella* SCAR markers (Table 1; SCARdb: <http://puma.icb.usp.br/eimeriaScardb/>, Fernandez *et al.* 2004) and the *E. maxima* microneme 1 gene (described previously, Blake *et al.* 2006)

were tested for their specificity against a panel of all seven *Eimeria* species that infect the chicken. Each PCR amplification was based upon a 20 µl volume consisting of 100 ng relevant genomic DNA template (eimerian or host), 1 µmol forward and reverse primers, 0.5 U *Taq* polymerase (Invitrogen), 10 mM Tris-HCl, 1.5 mM MgCl₂, 50 mM KCl and 0.2 mM all four dNTPs. Reaction conditions were 1 cycle: 94°C, 1 min, 30 cycles: 94°C, 1 min, 56°C, 1 min, 72°C, 1 min, 1 cycle: 72°C, 10 min. Primers targeting the chicken GAPDH gene and conserved regions of the eimerian 5S rDNA repeat were utilised for positive control (Myers *et al.* 2003; Blake *et al.* 2006). All PCR products were cloned using the pGEM Teasy vector system in XL1-Blue MRF competent cells, minipreped and sequenced as described by the relevant manufacturers (Promega, Southampton, UK; Stratagene, Cedar Creek, USA; Qiagen, Crawley, UK; Ceq 8000 Genetic Analysis System, Beckman Coulter (UK) Ltd., High Wycombe, UK, respectively).

Confirmation that the *E. maxima* MIC1 amplicon targeted by the quantitative PCR is representative of the species was obtained by sequencing a 185 bp fragment from the H and Weybridge (W) *E. maxima* strains. *E. maxima* W strain genomic DNA preparation and the PCR reactions were as described above. PCR products were cloned, prepared and sequenced as described above.

DNA dilution series. A panel of 13 different tenfold serial dilution series were created as the basis for these trials. Initially, series presenting 10⁶-10⁻¹ *E. acervulina*, *E. maxima*, *E. necatrix* or *E. tenella* genomes per 5 µl were prepared, using glycogen as a carrier (33 µg/ml, Larsen *et al.* 2002). Subsequently, a single 'combined' series representing 10⁵-10⁻¹ copies of all four *Eimeria* species genomes per 5 µl was made, together with individual series presenting 10²-10⁰ *E. acervulina*, *E. maxima*, *E.*

necatrix or *E. tenella* genomes in the presence of 10^4 copies of the three genomes not being diluted and 10^6 copies of the host chicken genome. In addition, pGEM Teasy plasmids harbouring the target of each species-specific real-time PCR were used as single copy template positive controls. Each plasmid, produced during the sequencing process described above, was linearised by *NdeI* digestion (Promega, Southampton, UK). Plasmid copy number was calculated (as described previously, Blake *et al.* 2006) and tenfold dilution series representing 10^8 - 10^{-1} copies of each produced as described above.

Real-time quantitative PCR. Real-time quantitative PCR was performed using the 7500 FAST Real-Time PCR System (Applied Biosystems) with the associated FAST Universal PCR Master Mix as described by the manufacturers. Briefly, each sample was amplified in triplicate, based upon a reaction containing 5 μ l DNA dilution, 500 nM forward and reverse primers and 250 nM probe. The Fast PCR conditions, modified from those suggested by the manufacturer, were 95°C for 20 seconds followed by 40 cycles of 95°C for 15 seconds and 60°C for 30 seconds. Data was collected at the end of each cycle. Replicate no template controls were incorporated into every assay. Negative control was provided by comparison between relevant dilution series.

Calculations and statistics. Triplicate cycle threshold data (Ct: the cycle number at which the fluorescence generated through probe cleavage exceeded the no template control) arising from real-time PCR of each DNA dilution were averaged and associated standard error of the mean calculated using Excel (Microsoft Excel 2002, Microsoft Corporation, 2001). An estimate of the number of PCR target copies

represented by Ct data was made by comparison with single copy plasmid DNA standard series. The efficiency of PCR amplification was determined as described elsewhere using the formula given below (Cummings and Tarleton 2003).

$$\text{Efficiency of PCR} = 10^{(-1/\text{slope})}$$

Results

Strain specificity of the *E. maxima* MIC1 amplicon. 185 bp fragments of the *E. maxima* MIC1 gene were PCR amplified from the H and W strains. Alignment with the MIC1 sequence published by Pasamontes *et al* (1993; GenBank M99058, strain not specified), revealed the absence of polymorphism.

***Eimeria* species-specific real-time PCR assay specificity.** Candidate PCR assays specific to *E. acervulina*, *E. maxima*, *E. necatrix* and *E. tenella* were first applied to a genomic DNA panel representing all seven species of *Eimeria* known to infect the chicken, together with the host as a background control (Figure 1). In each example a single product unique to the target species investigated was amplified (amplicon size given in Table 1).

When used in quantitative real-time PCR all four assays were shown to be highly reproducible, characterised by coefficient of regression values in excess of 0.99 over at least six orders of magnitude (eight using plasmid series) and average standard error of the mean values ≤ 0.15 (Table 2 and Figure 2). A comparable efficiency of PCR was calculated from each assay (Table 2). Comparison between

single- and multi-species dilution series clearly demonstrated that each species-specific assay was unaffected by the presence of genomic DNA derived from other *Eimeria* species (Figure 2). Indeed, even at the lower limits of detection each assay was unaffected by the background presence of non-target *Eimeria* species and host genomic DNA at far higher concentrations (up to three and five orders of magnitude higher respectively, Figure 3).

Eimeria species-specific real-time PCR assay sensitivity. Replicate real-time PCR analysis of each dilution series revealed the limit of detection for all four species-specific assays to be between 1 and 10 parasite genomes (Table 2 and Figure 2). Comparisons between the *E. maxima* specific assay (known to target the single copy MIC1 gene, Blake *et al.* 2006) and the other genomic and single copy plasmid DNA series suggests each PCR target to be present as a single copy within the relevant species genome (Figure 2).

Discussion

The influence of variables including host genotype, immune status, or chemical exposure on eimerian replication have hitherto been quantified using measures such as oocyst excretion, lesion scoring and host weight gain and food conversion.

Unfortunately, none of these measures can provide sensitive, quantitative species-specific data, irrespective of life cycle stage under examination. The work reported here describes the development and validation of quantitative real-time PCR assays capable of specifically enumerating the four most economically important *Eimeria*

species. These assays supplement existing single and multiplex standard PCR assays for identification (Schnitzler *et al.* 1999; Fernandez *et al.* 2003) and quantitative assays for two species not shown to be species-specific (Blake *et al.* 2006; Swinkels *et al.* 2006). All four assays have been shown to be directly comparable in terms of sensitivity and robustness, capable of detecting ten parasite genomes but not one, unaffected by the presence of DNA derived from the host or the other test species. Every sporulated oocyst contains eight eimerian genomes, suggesting that the DNA equivalent of a single oocyst will be consistently detectable given normal experimental replication (between one and ten genomes detected per reaction). Mature intracellular stages represent in the order of 10-100 eimerian genomes (depending on species and stage; Johnston *et al.* 2001); suggesting that even a fraction of one may be counted. None of the assays were overwhelmed within the concentration ranges tested (single copy targets up to and including 10^8 copies). The validated species-specificity of each assay, together with the comparable limits of detection and efficiency of PCR, will facilitate comparison between species, for example in field or co-infection trials. The inclusion of a generic *Eimeria* species quantitative assay (for example targeting the 5S ribosomal repeat, Blake *et al.* 2006) will compliment the assays described here. Calculation of the total numbers of eimerian parasites present will either corroborate the sum of the species-specific assays or reveal whether species other than those targeted are present at a significant level. Given suitable apparatus two or more of the assays reported here could be multiplexed. Indeed, the establishment of technology supporting multiplexed quantitative PCR assays for four or more distinct targets (Kirs and Smith 2007) could be used to test for all four species, plus a host standard, in a single assay.

A feature of many *Eimeria* species, most notably *E. maxima* and to a lesser extent *E. acervulina*, is genetic (and immunological) variation between strains (Joyner, 1969; Barta *et al.* 1998). The SCAR markers selected for use as *Eimeria* species-specific quantitative PCR targets in this study have previously been shown to be both species-specific and to characterise all strains tested (*E. acervulina*: six strains tested, *E. necatrix*: 2, *E. tenella*: 5; <http://puma.icb.usp.br/eimeriaScardb/>, Fernandez *et al.* 2004). Additional *E. acervulina* and *E. necatrix* strains (one of each) were sequenced during this study (data not shown). The *E. maxima* PCR target described here, a fragment of the MIC1 gene, was found to be identical in the strain from which the sequence was originally defined (Pasamontes *et al.* 1993) and in the H and W *E. maxima* strains, previously shown to exhibit significant genetic and phenotypic polymorphism (Blake *et al.* 2004).

Eimeria species-specific real-time PCR assays may be applied to both species discrimination and enumeration throughout the environmental and intracellular stages of the parasite life cycle. The limits of detection described here suggest that sensitivity in enumerating the oocyst content of a sample will only be constrained by the quality of oocyst recovery and DNA extraction. Separation based upon specific gravity (e.g. salt flotation) supports efficient oocyst recovery from environmental samples containing very small numbers of oocysts (Shirley and Harvey 1996). PCR targeting the ITS1 repeat (140 copies per *E. tenella* genome) has been shown to be capable of detecting 10^2 purified *Eimeria* oocysts or 10^4 oocysts gram^{-1} faecal material (Jenkins *et al.* 2006). The oocyst content of litter samples from commercial broiler production is likely to surpass such levels by the time the chickens are 7-14 days old (Williams 2002). Whilst the PCR targets validated in this study are single copy, the use of triplicate testing as standard and the extra cycles of amplification used (40 compared

to 35), suggest that a similar level of detection is achievable in field samples. It is important to note that the degree of oocyst sporulation in a faecal or litter sample, or the efficiency of recovery, will affect sensitivity and interpretation of the result. Nonetheless, the ability to quantify and unequivocally discriminate between DNA from different *Eimeria* species offers a considerable advantage over traditional microscopic techniques. In addition to surveillance/detection and laboratory use *Eimeria* species-specific quantitative PCR provides the opportunity to accurately formulate consistent batches of multi-valent live parasite vaccines and monitor viability (for example by comparison of parasite numbers before and after excystation and sporozoite purification).

The validation of genuinely specific quantitative real-time PCR assays for four commercially important *Eimeria* species has established a key set of tools with value to practical poultry husbandry, commercial vaccine delivery and scientific research. The influence of host, management and other environmental factors on eimerian reproduction may now be quantified using a robust, quick assay, irrespective of the life cycle stage under investigation or the presence of other, non-target pathogens.

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Table 1. Primer/probe combinations used in *Eimeria* species-specific quantitative real-time PCR.

| Oligonucleotide name | Oligonucleotide identity | Sequence (5'-3') | Sequence source ^a (amplicon size) |
|----------------------|--------------------------------|--|---|
| Eac_qPCRf | <i>E. acervulina</i> : forward | CTCGCGTGTTCAGCACTACAT | Ac-R01-1731 |
| Eac_qPCRr | <i>E. acervulina</i> : reverse | GATACGCTGCTTTGCCTTTC | (124 bp) |
| Eac_qPCRp | <i>E. acervulina</i> : probe | Fam-TAAGCAACGGGCTACAACCTCACCGTAT-Tamra | |
| Ema_qPCRf | <i>E. maxima</i> : forward | TCGTTGCATTTCGACAGATTC | Blake et al., (2006) |
| Ema_qPCRr | <i>E. maxima</i> : reverse | TAGCGACTGCTCAAGGGTTT | (138 bp) |
| Ema_qPCRp | <i>E. maxima</i> : probe | Fam-ATTGTCCAGCCAAGGTTCCCTTCG-Tamra | |
| Ene_qPCRf | <i>E. necatrix</i> : forward | AACGCCGGTATGCCTCGTCG | Nc-AD10-702 |
| Ene_qPCRr | <i>E. necatrix</i> : reverse | GTACTGGTGCCAACGGAGA | (141 bp) |
| Ene_qPCRp | <i>E. necatrix</i> : probe | Fam-CCGTAGCATAGCTCAGGCAGCCAC-Tamra | |
| Ete_qPCRf | <i>E. tenella</i> : forward | TCGTCTTTGGCTGGCTATTC | Tn-E03-1161 |
| Ete_qPCRr | <i>E. tenella</i> : reverse | CAGAGAGTCGCCGTCACAGT | (121 bp) |
| Ete_qPCRp | <i>E. tenella</i> : probe | Fam-CTGGAAAGCGTCTCCTTCAATGCG-Tamra | |

^a*Eimeria* species-specific primers and probes were designed from sequenced SCAR data (<http://puma.icb.usp.br/eimeriaScardb/>, Fernandez *et al.* 2004) with the exception of *E. maxima*, where the primer probe set was as described previously (Blake *et al.* 2006).

Table 2. Quantitative real-time PCR standard dilution series data.

| Species | Template ^a | Genome/plasmid copy no. | | Coefficient of regression ^d | Efficiency of PCR | Average SEM |
|----------------------|-----------------------|-----------------------------------|----------------------------------|--|-------------------|-------------|
| | | Range tested ^b | Linear range ^c | | | |
| <i>E. acervulina</i> | Genomic DNA | 10 ⁶ -10 ⁻¹ | 10 ⁶ -10 ¹ | 0.9987 | 0.527 | 0.15 |
| | Plasmid DNA | 10 ⁸ -10 ⁻¹ | 10 ⁸ -10 ¹ | 0.9991 | ns | 0.11 |
| | Mixed genomic DNA | 10 ⁵ -10 ⁻¹ | 10 ⁵ -10 ¹ | 0.9989 | 0.523 | 0.12 |
| <i>E. maxima</i> | Genomic DNA | 10 ⁶ -10 ⁻¹ | 10 ⁶ -10 ¹ | 0.9995 | 0.514 | 0.08 |
| | Plasmid DNA | 10 ⁸ -10 ⁻¹ | 10 ⁸ -10 ¹ | 0.9996 | ns | 0.12 |
| | Mixed genomic DNA | 10 ⁵ -10 ⁻¹ | 10 ⁵ -10 ¹ | 0.9995 | 0.513 | 0.13 |
| <i>E. necatrix</i> | Genomic DNA | 10 ⁶ -10 ⁻¹ | 10 ⁶ -10 ¹ | 0.9991 | 0.512 | 0.10 |
| | Plasmid DNA | 10 ⁸ -10 ⁻¹ | 10 ⁸ -10 ¹ | 0.9998 | ns | 0.09 |
| | Mixed genomic DNA | 10 ⁵ -10 ⁻¹ | 10 ⁵ -10 ¹ | 0.9987 | 0.523 | 0.12 |
| <i>E. tenella</i> | Genomic DNA | 10 ⁶ -10 ⁻¹ | 10 ⁶ -10 ¹ | 0.9996 | 0.514 | 0.09 |
| | Plasmid DNA | 10 ⁸ -10 ⁻¹ | 10 ⁸ -10 ¹ | 0.9946 | ns | 0.13 |
| | Mixed genomic DNA | 10 ⁵ -10 ⁻¹ | 10 ⁵ -10 ¹ | 0.9984 | 0.513 | 0.07 |

^aTemplate DNA consisted of total genomic DNA (single species or an equal mix of all four species) or a single copy of the target in pGEM Teasy (plasmid).

^bGenome/plasmid copy number calculated based upon genome/plasmid size compared with DNA concentration and *Eimeria*-generic real-time PCR (Blake *et al.* 2006).

^cThe genome/plasmid copy number range from which the linear phase of the standard curve was amplified by real-time PCR. Where a mix of four species was used each individual species was represented to the given concentration.

^dValues derived from the linear range given in the preceding column.

ns = not shown.

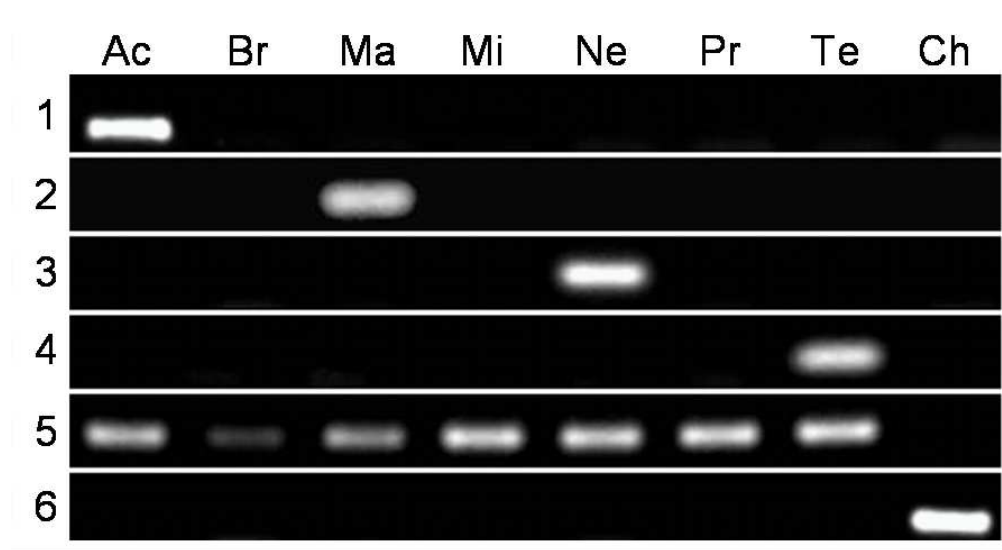
Captions to Figures

Figure 1. Discriminatory ability of *Eimeria* species-specific PCR.

Columns: Ac, *E. acervulina*, Br, *E. brunetti*, Ma, *E. maxima*, Mi, *E. mitis*, Ne, *E. necatrix*, Pr, *E. praecox*, Te, *E. tenella*, Ch, chicken. Rows: 1, Ac-specific, 2, Ma-specific, 3, Ne-specific, 4, Te-specific, 5, *Eimeria*-specific (5S rRNA gene; Blake *et al.* 2006), 6, host-specific (chicken GAPDH gene; Myers *et al.* 2003).

Figure 2. Quantitative real-time PCR standard curves from four *Eimeria* species-specific assays. Ten-fold serial dilution series of pure genomic DNA (●), mixed genomic DNA (○, all four species equally represented) or single copy plasmid control template (□) were tested to establish each assay's sensitivity, reproducibility, linear range and efficiency (Table 2). A, *E. acervulina*, B, *E. maxima*, C, *E. necatrix*, D, *E. tenella*. Ct = cycle threshold.

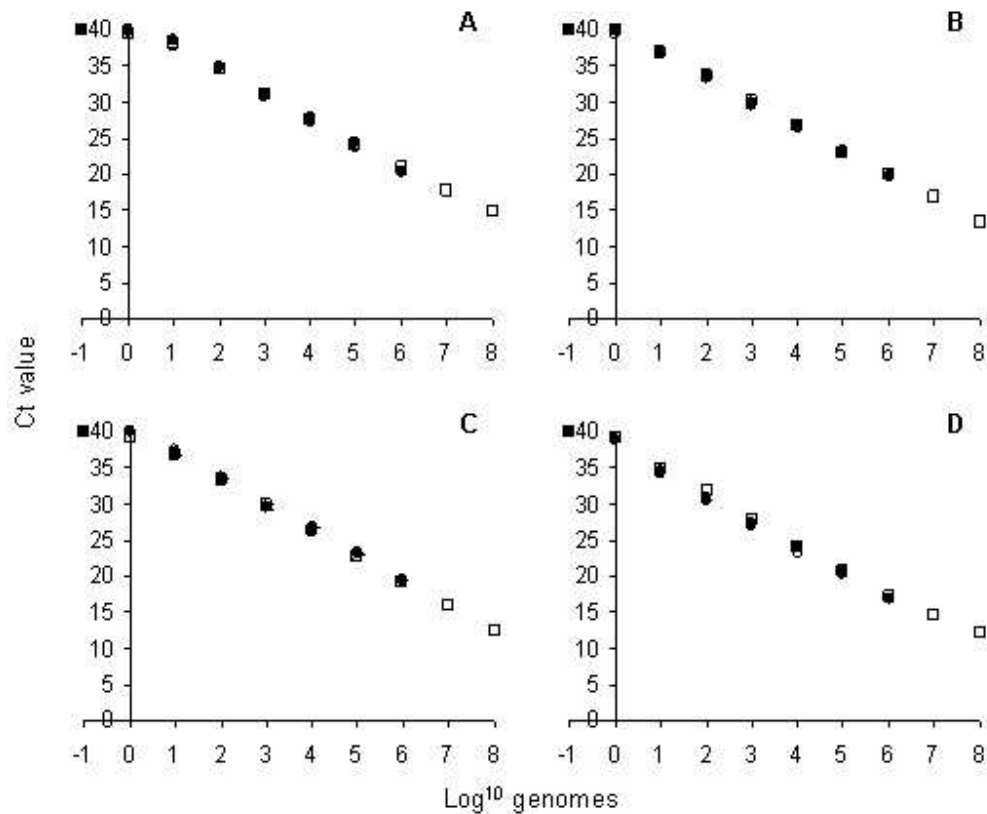
Figure 3. The influence of excess non-target DNA on four *Eimeria* species-specific quantitative real-time PCR assays at the lower limit of detection. Ten-fold serial dilution series of genomic DNA extracted from the target *Eimeria* species with (○) or without (●) 10^4 copies of each of the other three species and 10^6 copies of the host chicken genome. A, *E. acervulina*, B, *E. maxima*, C, *E. necatrix*, D, *E. tenella*. Ct, cycle threshold.



Discriminatory ability of *Eimeria* species-specific PCR.

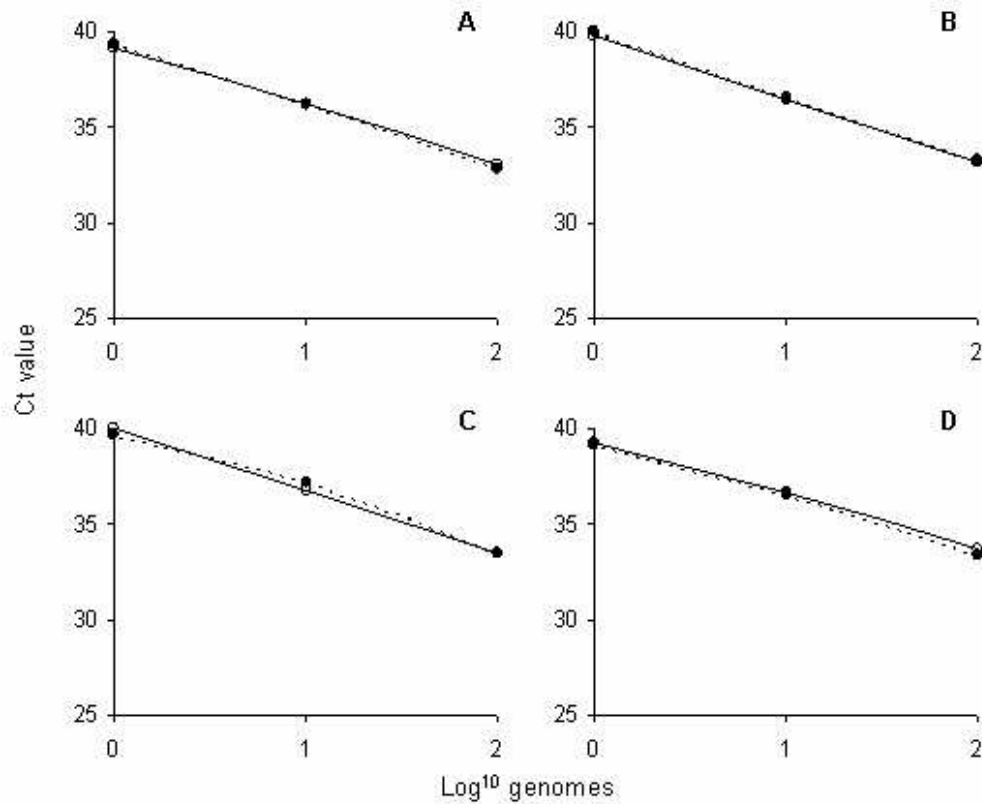
Columns: Ac = *E. acervulina*, Br = *E. brunetti*, Ma = *E. maxima*, Mi = *E. mitis*, Ne = *E. necatrix*, Pr = *E. praecox*, Te = *E. tenella*, Ch = chicken. Rows: 1 = Ac specific, 2 = Ma specific, 3 = Ne specific, 4 = Te specific, 5 = *Eimeria* specific (5S rRNA gene; Blake *et al.* 2006), 6 = host specific (chicken GAPDH gene; Myers *et al.* 2003).

151x82mm (150 x 150 DPI)



Quantitative real-time PCR standard curves from four *Eimeria* species-specific assays. Tenfold serial dilution series of pure genomic DNA (●), mixed genomic DNA (○, all four species equally represented) or single copy plasmid control template (□) were tested to establish each assay's sensitivity, reproducibility, linear range and efficiency (Table 2).

A = *E. acervulina*, B = *E. maxima*, C = *E. necatrix*, D = *E. tenella*. Ct = cycle threshold.
135x112mm (96 x 96 DPI)



The influence of excess non-target DNA on four *Eimeria* species-specific quantitative real-time PCR assays at the lower limit of detection. Tenfold serial dilution series of genomic DNA extracted from the target *Eimeria* species with (○) or without (●) 104 copies of each of the other three species and 106 copies of the host chicken genome.

A = *E. acervulina*, B = *E. maxima*, C = *E. necatrix*, D = *E. tenella*. Ct = cycle threshold.

136x111mm (96 x 96 DPI)

only