

USE OF A SEQUENCE CHARACTERIZED AMPLIFIED REGIONS (SCARS) – MULTIPLEX PCR METHOD TO IDENTIFY *EIMERIA* SPECIES OF CHICKENS FROM WIDELY DISTRIBUTED GEOGRAPHIC AREAS

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Abstract

A Random Amplified Polymorphic DNA-Sequence Characterized Amplified Regions (RAPD-SCAR) marker based Multiplex-Polymerase Chain Reaction (PCR) was evaluated as a potential method of differentiating species of *Eimeria* oocysts in chickens. Oocysts of chicken coccidia were obtained from different geographical areas of 3 continents (Africa, North America and Europe) and genomic DNA was extracted. PCR was performed using pairs of RAPD-SCAR based primers that specifically amplify genomic DNA from six species of *Eimeria* found in chickens (*E. maxima*, *E. tenella*, *E. acervulina*, *E. mitis*, *E. necatrix* and *E. brunetti*) in a single reaction tube. Results showed that the method is useful in differentiating species of coccidia obtained from Nigeria, the United Kingdom, United States of America and Canada. Samples containing more than one species of *Eimeria* were successfully detected by the method despite low numbers of oocysts in some of the samples (less than 1000 oocysts per ml of isolate). The method demonstrates the simultaneous detection of the 6 *Eimeria* species affecting chickens in a single-tube multiplex PCR either as single or mixed infections; and offers a universal tool for the epidemiological study of coccidiosis in poultry.

Introduction

Coccidiosis is caused by intracellular parasitic protozoa of the genus *Eimeria* with infections frequently presented as simultaneous infections of multiple species (Barta, 2001). In chickens, seven *Eimeria* species are described and each displays unique pathogenicity. They include: *E. acervulina*, *E. brunetti*, *E. maxima*, *E. tenella*,

E. mitis, *E. praecox* and *E. necatrix*. These obligate parasites affect the intestinal mucosa and cause lesions of variable extent and severity (McDougald & Reid, 1997).

Coccidiosis in Nigeria remains a limiting health problem in intensive poultry husbandry, Halle *et al.* (1998); Majaro, (1980; 1981) and Etuk *et al.* (2004) reported moderate to high prevalence

(36-43%) of the disease in poultry. Coccidiosis is particularly important in the tropical environment (Seifert, 1996); and control measures are still predominantly based on the use of anticoccidial drugs in feeds (Fernandez *et al.*; 2003). The increasing resistance to many anticoccidials is likely to limit their long term use in control strategy in future (Chapman, 1999). Besides, cost of control has been described as the basis for its economic importance (Majaro, 1980; 1981). Methods of differentiating among *Eimeria* species have mostly been based on biological features of size, colour and shape of oocysts, predilection sites and nature of their endogenous stages. However, the reliable identification of *Eimeria* to species level using this method is frequently not possible (Barta, 2001). Oocysts of *Eimeria* vary in size with *E. maxima* being the largest ($20 \times 30 \mu$) and *E. mitis* the smallest ($14 \times 16 \mu$) but many species overlap significantly in their dimensions. This wide range of oocysts sizes and their identical shapes usually make it difficult to morphologically distinguish among oocysts of various *Eimeria* species using the compound microscope (Allen & Fetterer, 2002). Besides, different species vary in their pathogenicity, drug resistance, and other biological parameters (Fernandez *et al.*; 2003) and the precise discrimination of different species and/or strains is important to epizootiological and population biology studies. Another important application of speciation is the quality control of parasites used to prepare live vaccines (Fernandez *et al.*; 2003).

Molecular methods used for Eimeriid Coccidia identification included isoenzyme analysis, DNA hybridization and random-amplified polymorphic (RAPD-PCR). Previous studies by Johnston and Fernando (1995; 1997) described extensively, the use of electrophoretic profiles of ten isoenzymes for *Eimeria* speciation and

detection of differences among *Eimeria* species. RAPD-PCR has been used for both species and strain differentiation (Fernandez *et al.*; 2003). Although, results were promising, isoenzyme methods required large numbers of oocysts, (usually requiring *in vivo* amplification of any sample), yielded limited number of variable enzymes, low levels of polymorphism and is considered too cumbersome and time-consuming. RAPD-PCR was difficult to standardize and adopt universally and it could not deal with mixed samples. Shirley, (1994) had earlier proposed the use of DNA hybridization for species and strains discrimination but for its complexity, its use was also impaired across different laboratories (Procunier *et al.*; 1993; Shirley 1994; Johnston & Fernando 1995; 1997; Fernandez *et al.*; 2003). Thus, molecular techniques using PCR of DNA extracted from the parasite is the current method recommended for species differentiations (Allen and Fetterer, 2002).

The use of multiplex PCR (Chamberlain *et al.*; 1988) enables the simultaneous amplification of more than one locus in the same reaction by using more than one pair of primers. Researchers have found the application of multiplex PCR useful in molecular characters and targets and in many areas of DNA testing which included detection of pathogens and genotyping applications where simultaneous analysis of multiple markers is required (Crisan, 1994; Mutirangum *et al.*; 1993). Fernandez *et al.*; (2003) reported the use of a novel sets of SCAR Makers isolated from RAPD fragments incorporated into a cost effective simple multiplex PCR that allowed simultaneous differentiation of all seven species of *Eimeria* affecting chicken and propose its effective universal usage where strains from different distinct geographical regions were tested. The test allows a 7-fold increase of the through-put

(Fernandez *et al.*; 2003) as compared to tests utilizing one-step PCR of individual species of the parasite; thus offering a potential universal tool for the epidemiological study of coccidiosis in poultry.

This study was therefore, aimed at evaluating the usefulness of multiplex PCR of the small subunit rRNA of *Eimeria* species of chicken in Nigeria developed from SCAR Makers for the first time and in simultaneous discrimination of species from two other different geographical sources of North America and Europe.

Materials and Methods

Sources of Oocysts

Oocysts isolates were obtained either as field faecal samples or mucosal scrapings from caecal lesion submitted to the National Veterinary Research Institute's Central Diagnostic Laboratories, (D) and the Parasitology Division, (P). Likewise, other samples were obtained from poultry farms located in Guelph, Ontario and Florida, US (G), while Houghton (H) strain of *E. tenella* originally derived from a field case of caecal coccidiosis in 1949, and obtained as pure lines.

Parasite Purification

Small samples of the faecal materials were homogenized in a blender and filtered through a 0.15 mm mesh sieve into a beaker. Filtrate was pelleted by centrifugation at 800g for 2 min. The supernatant was discarded and pellets were re-suspended in distilled water. To collect oocysts directly from the caeca, mucosal scrapings were made from the caecal lesion and washed into a beaker, or entire pieces of the caeca were ground in a blender with 2.5% (w/v) potassium dichromate solution to release the unsporulated oocysts. The tissue suspension was filtered through 0.15 mm mesh sieve and

filtrates were pelleted by centrifugation at 800rpm for 2 minutes. Oocysts were finally recovered from pellets by adding saturated sodium chloride (aqueous) to pellets, shaken vigorously; the mixture was then centrifuged at 400g for 10-15 min. The oocysts were recovered by decanting the upper portion of the supernatant, diluting at least 10-fold with distilled water and again centrifuged. The pelleted oocysts were washed, four times by mixing the pellet in water, centrifuging to pellet the oocysts, decanting the supernatant and repeating to remove the salt solution. The salt-free oocysts were then stored in 2.5% potassium dichromate solution at 4°C (Mac Dougald *et al.*; 1997).

DNA Extraction

One ml of washed oocyst isolate was centrifuged at 11,000g for 2 minutes. The supernatant was discarded and the pellet was covered with 100µl PBS to which sterile glass beads (250µm diameter) from Biospec Products Inc (Bartlesville, OK, USA) were added up to the 250µl mark on the graduated 1.5ml micro centrifuge tube (Fisher Scientific USA). Sporocysts and sporozoites were released from oocysts by vortexing for 4 minutes in the presence of glass beads. After vortexing, DNA extraction was carried out using DNAzol (GIBCO BRL Life Technologies, USA): 1 ml of DNAzol reagent was added to the 250 µl glass bead/oocyst lysate mixture. The extraction was allowed to proceed at room temperature for 30 min with mixing by inversion every 2 minutes. After extraction, the tubes were centrifuged at 11,000g for 15min at 4°C to pellet the glass beads and non-solubilized oocyst material. The supernatant was collected into new 1.5ml microfuge tubes and DNA was precipitated adding 500µl of 100% (absolute) ethanol (warmed to room temperature) followed by centrifugation at 11,000g for 5min at 4°C.

Pelleted DNA was washed with 500µl of ice-cold 70% ethanol and allowed to stand at room temperature inverted on filter paper with the tube open to permit the alcohol to evaporate. The dried pellet was dissolved into 50µl of molecular biology grade water by gentle heating at 40°C. Solubilized DNA was stored at 4°C for immediate use and -20°C for longer term storage. DNA quality and quantity was estimated visually under UV illumination following electrophoresis on a 2% agarose gel and stained with ethidium bromide.

Multiplex PCR and Identification of the PCR Products

Multiplex PCR reaction amplification was performed in 25µl total volumes and included 12.5µl of premix, 10.5µl Nuclease Free Water, and 2µl Template DNA. Typically the premix consisted of the following: 100 µM dNTP, 50mM MgCl₂, 10x MgCl₂ Buffer, 5U of Invitrogen DNA Taq Polymerase; primer concentrations 10µM for each primer pair of Ac-01-F, Ac-01-R (bp 811); Br-01-F, Br-01-R (bp 626); Tn-01-F Tn-01-R (bp549); Mt-01-F, Mt-01-R (bp460); Pr-01-F, Pr-01-R (bp354); Mx-01-F, Mx-01-R (bp272) and Nc-01-F, Nc-01-R(bp200) were used. Cycling conditions consisted of an initial denaturation at 96 °C for 6min and 40cycles of 1 min at 94°C and 2min at 55 °C, with a final extension step at 72 °C for 5min. Amplification reactions were performed with a PTC 1148(100-240 V) MJ Mini Gradient Thermal Cycler (BIO RAD California, USA). Amplicons produced were analysed by separation on 2% agarose gel stained with ethidium bromide and visualized under UV light.

Results and Discussion

The specificity and sensitivity of these SCAR markers primers have been determined earlier

by Fernandez *et al*; (2003) and confirmed in this study (Figure 3). This report confirms the effectiveness on *Eimeria* species from different geographical origins; the 7 primer pairs used consistently presented a species-specific pattern of amplified DNA fragments using the optimized multiplex PCR conditions previously reported by Fernandez *et al*; (2003). A total of 6 *Eimeria* species, *E. acervulina* bp 811; *E. brunetti*, bp 626; *E. tenella* bp539; *E. mitis*, bp 460; *E. maxima*, bp 272 and *E. necatrix* bp200, were identified by multiplex PCR in various field samples (Figures 1 and 2). Most field samples revealed multiple species of *Eimeria* demonstrating that it is possible to detect multiple infections simultaneously in a single-tube reaction.

This study confirms the possibility of differentiating coccidia oocysts in a sample containing one or more species using simultaneous detection of up to 7 *Eimeria* species affecting chickens using a single-tube SCAR-based multiplex PCR; and confirming its merit with respect to economy of consumables and labour.

The development of Sequence-Characterised Amplified Regions (SCAR) Makers, first described by Paran and Micheltore (1993) derived from random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) in which specific fingerprints were generated and re-amplified under strictly controlled conditions by pairs of specific primers; worked effectively in detecting and discriminating all *Eimeria* species of chicken. Fernandez *et al*; (2003) identified RAPD-SCAR markers unique to each *Eimeria* species infecting chickens and from these generated an economical multiplex PCR that allowed simultaneous differentiation of all seven *Eimeria* species affecting chicken. These authors also reported its effective

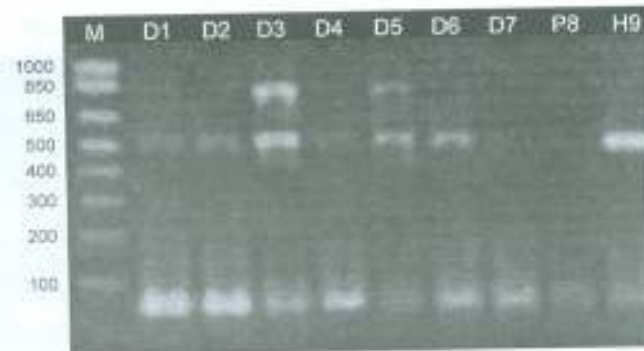


Figure 1: Agarose gel electrophoresis of multiplex PCR products using SCAR Markers from various fecal samples obtained from Nigeria (D& P samples) and Houghton (H sample); (M) DNA size marker (base pairs [bp], scale to left), (D1) *E. tenella* 539 bp, (D2) *E. tenella* 539 bp, (D3) *E. acervulina* 811 bp, *E. tenella* 539 bp, *E. mitis* 460 bp, (D4) *E. acervulina* 811 bp (weak), *E. tenella* 539 bp; (D5) *E. acervulina* 811 bp, *E. tenella* 539 bp, *E. mitis* 460 bp (weak); (D6) *E. acervulina* 811 bp (weak), *E. tenella* 539 bp; (D7) Negative, (P8) Negative Template Control; (H9) Houghton Strain *E. tenella* 539 bp (positive control).

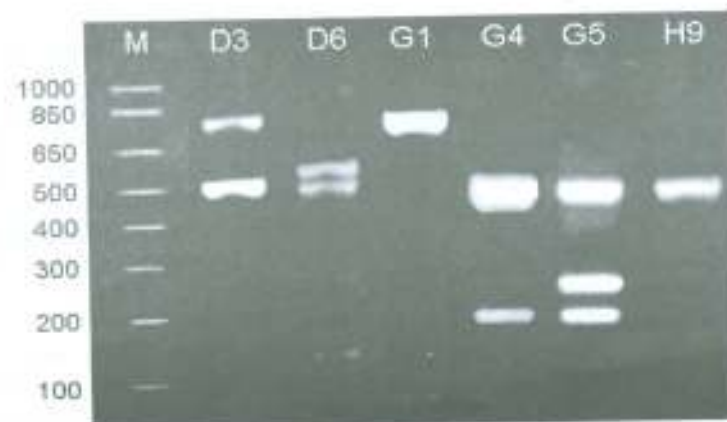


Figure 2: Agarose gel electrophoresis of multiplex PCR products using SCAR Markers; from various fecal samples obtained from Guelph (G samples); Nigeria (D samples); (M) DNA size marker (base pairs [bp], scale to left), (D3) *E. tenella* 539 bp, *E. acervulina* 811 bp; (D6) *E. brunetti* 626 bp, *E. tenella* 539 bp; (G1) *E. acervulina* 811 bp; (G4) *E. tenella* 539 bp, *E. necatrix* 200 bp; (G5) *E. tenella* 539 bp, *E. maxima* 271 bp, *E. necatrix* 200 bp; (H9) Houghton Strain *E. tenella* 539 bp (positive control).



Figure 3: Agarose gel electrophoresis of multiplex PCR products using SCAR Markers; (M) DNA size marker (base pairs [bp], scale to right) with 6 individual *Eimeria* species of chickens (Lanes 1 - 6) or 5 *Eimeria* species in a single sample (Lane 7). Lanes: (M) DNA size marker (base pairs [bp], scale to left); 1) *E. acervulina* 811 bp; 2) *E. brunetti* 626 bp; 3) *E. tenella* 539 bp; 4) *E. mitis* 460 bp; 5) *E. maxima* 271 bp; 6) *E. necatrix* 200 bp; 7) mixture of 5 *Eimeria* species; 8) negative control.

application for species discrimination from two different continents. Similarly, SCAR markers have been used in molecular diagnosis of *Cryptosporidium parvum* (Morgan *et al*; 1996) and *Trypanosoma cruzi* differentiation (Brisse *et al*; 2000).

The SCAR-based multiplex PCR in this study, was able to differentiate *Eimeria* species from all regions from which samples were obtained. We tested samples from 3 different continents (Africa, Europe and North America) and suggest that SCAR-based multiplex PCR offers a potential universal tool for the epidemiological study of coccidiosis in poultry. Our trials confirmed and agreed with the observations of Fernandez *et al*; (2003) that SCAR-based multiplex PCR was not apparently affected by internal variations within species and may be applicable worldwide. However, SCAR-based multiplex PCR may be limited in

terms of differentiating among strains of *Eimeria* species. We observed some sequence differences within multiplex PCR-generated amplification products from distinct strains of the same *Eimeria* species [data not shown]. Sequence identity between sequences submitted by Fernandez *et al*; (2003) ranged from 98.9 to 100% depending on the *Eimeria* species examined.

Therefore more work needs to be done to determine if sequences obtained from multiplex PCR-generated amplification products could be used for strain differentiation. Any sequence variability within species-specific PCR products could be extremely useful in the quality control of live vaccine formulations. However, sequence variation within amplicons may confound attempts to apply real time PCR technology to this multiplex PCR method if this sequence variation affected the melting

temperature of the resulting PCR amplicons. Other methods of detection and discrimination of multiple *Eimeria* species using the internal transcribed spacer 1 (ITS1) region of nuclear ribosomal DNA (rDNA) have been described (Schnitzler et al 1998; Jenkins et al, 2006). RAPD-PCR (Procunier et al 1993) and isoenzymes analysis (Shirley, 1975; Johnston & Fernando, 1997) have also been demonstrated to be effective in characterizing *Eimeria* species but are not useful for identification of mixed *Eimeria* species in a sample. Isoenzyme analysis requires large amounts of parasite material of both the test and reference strains of the parasites.

The added advantage of being able to detect very low numbers of oocysts in a sample was demonstrated by Fernandez et al (2003) and confirmed in our study. Using titrated concentrations of oocysts, simultaneous detection was possible for samples containing as few as 2-8 sporulated oocysts for most of the species. Samples containing about 100 sporulated oocysts could be reliably amplified using the multiplex PCR method (Fig. 3). In our test, we used DNA samples of *E. tenella* obtained as pure line and mixed isolates from the field with no marked difference in quality of bands obtained. (Fig. 2) Fernandez et al; (2003) had earlier subjected the reliability and robustness of the assay to varying DNA concentrations, different models of thermocyclers and different sources of polymerase enzymes and concluded that the assay may be performed in different laboratories and produce comparable results. This study confirms the multiplex PCR to be simple and reproducible worldwide being able to detect *Eimeria* oocysts from different regions of the world. We therefore conclude that the method could complement morphological speciation of *Eimeria* species infecting

chickens, especially in regions where large scale studies are required with minimal resources. With the encouraging results obtained in this study, we recommend development of similar multiplex PCR methods for detecting coccidiosis in other intensively reared birds such as turkeys (Obaso & Ngbagu 2000). The multiplex PCR method could be useful for quality control in live oocyst vaccine production, monitoring of infection in poultry establishments and perhaps as a means of determining the success of prophylactic and/or therapeutic use of anticoccidial drugs.

To the best of our knowledge, this report is the first on the use of SCAR Markers in the multiplex -PCR diagnostic assay of *Eimeria* species in chicken to include samples from Nigeria.

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