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Short Communication

Development of a tetra-primer ARMS-PCR for detecting the E198A SNP in the isotype-1 β-tubulin gene of *Haemonchus contortus* populations in China

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ABSTRACT

The tetra-primer ARMS-PCR is a rapid, simple and low cost method for single nucleotide polymorphism (SNP) genotyping and has been used to detect SNPs associated with diseases and drug resistance. E198A in the isotype-1 β-tubulin gene is one of the three SNPs associated with benzimidazole resistance in parasitic nematode *Haemonchus contortus*. However, up to now, only PCR-RFLP method was used to test E198A in *H. contortus*. In the present study, we developed a tetra-primer ARMS-PCR to detect E198A in *H. contortus* and the accuracy of the results was compared with that of PCR-coupled sequencing. The results showed that optimization of PCR reaction system, especially the proportion of the amount of inner and outer primers, could achieve desirable amplification effect. Three different profiles displaying three distinct genotypes could be identified clearly and intuitively on the agarose gel where the samples with amplified PCR products containing two bands of 433 bp and 200 bp in size indicated susceptible homozygous (SS), those with PCR products containing two bands of 433 bp and 284 bp in length indicated resistant homozygous (RR) and the samples with amplified PCR products containing three bands of 433 bp, 284 bp and 200 bp in size indicated heterozygous (RS). The results showed that the established method can be successfully applied to the detection of E198A in *H. contortus*, which has high accuracy and is easy to perform.

1. Introduction

Gastrointestinal nematodes cause severe parasitic diseases to domestic ruminants, leading to serious production and economic losses. One of these parasites is *Haemonchus contortus*, which is almost the most prevalent and pathogenic trichostrongyloid nematode worldwide. Since the production of thiabendazole in 1961 (Brown et al., 1961), benzimidazole-based anthelmintics have been used for several decades to control *H. contortus* and related gastrointestinal nematodes. The excessive and long-term use of these anthelmintics has resulted in the selection of resistant parasites (Kotze et al., 2014).

Studies have identified that benzimidazole (BZ) elicit effects through the binding with β-tubulin to block the formation of microtubules in nematodes (Kohler, 2001). Three different single nucleotide polymorphisms (SNPs) in the isotype-1 β-tubulin gene known as F167Y (TTC to TAC) (Silvestre and Cabaret, 2002), E198A (GAA to GCA) (Ghisi et al., 2007; Rufener et al., 2009) and F200Y (TTC to TAC) (Kwa et al., 1994, 1995) have been associated with BZ resistance in *H. contortus*. The mutated nucleotide sequence has led to the change of protein structure which reduced the binding affinity of BZ molecules to β-tubulin (Prichard, 2001). Up to now, F200Y is still the primary SNP with respect to BZ resistance in most countries with high frequency. E198A and F167Y are both less frequent with a small scale distribution around the world (Kotze et al., 2014). However, in a recent report, E198A was identified among six of the eight *H. contortus* populations and occurred more frequently than did F200Y in China. Meanwhile, F167Y was absent in all populations. Hence, we concluded that it is urgent to develop molecular approaches to detect E198A in field populations of *H. contortus* in China (Zhang et al., 2016).

A number of molecular methods have been developed to assess the BZ resistance such as pyrosequencing and a variety of PCR-based approaches (von Samson-Himmelstjerna et al., 2007, 2009). Since the great cost and need of special equipment to perform pyrosequencing, it is not appropriate to be used widely in the field. Although PCR-based techniques have been developed, most of them were just focused on F200Y (von Samson-Himmelstjerna et al., 2007). As far as we know, only a PCR-RFLP method (Ghisi et al., 2007) and an allele-specific PCR (Rufener et al., 2009) were developed to test the E198A in the isotype-1 β-tubulin gene in *H. contortus*. Here we established a PCR method based on amplification refractory mutation system (tetra-primer ARMS-PCR)

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(Ye et al., 2001), which uses four primers in one reaction to distinguish three genotypes at codon 198 (homozygous susceptible, SS; heterozygous, RS and homozygous resistant, RR) and compared with the sequencing results of the same samples from our previous study (Zhang et al., 2016).

2. Materials and methods

2.1. Parasite populations and genomic DNA isolation

The animal procedures were approved by the Committee on the Ethics of Animal Experiments of Hubei Province, China (Permit Number: SYXK-0029). For all *H. contortus* populations (HuB, HeB, YN, SX, GX, IM, LN and HLJ), the approach of genomic DNA isolation from single worm and the data of PCR-coupled sequencing have all been described in previous studies (Gasser et al., 1993; Zhang et al., 2016). The brief description of DNA isolation and PCR-coupled sequencing are as follows. The total genomic DNA was extracted from individual adults using sodium dodecyl-sulfate/proteinase K treatment, followed by spin-column purification (Wizard DNA Clean-Up, Promega) and for further test, we have adjusted the concentration of each genomic DNA sample to 40 ng/μL using Nanodrop 2000 Spectrophotometer. The amplicons of each PCR were column-purified (Wizard PCR-Preps, Promega) and then directly sequenced (in separate reactions) with corresponding primers using the BigDye Terminator v.3.1 cycle sequencing kit (Applied Biosystems) in an automated sequencer (PRISM3730, ABI).

2.2. Primers design

Based on the sequence of isotype-1 β-tubulin gene of *H. contortus* available in the NCBI database (GenBank accession no. X67489), the tetra-primer ARMS-PCR primers were designed using an online program at http:// cedar.genetics.soton.ac.uk/public_html/primer1.html (Ye et al., 2001) and the primer sequences are shown in Table 1. The other primer pair F1 (5′-GGAACAATGGACTCTGTTCG-3′) and R1 (5′-GAATCGAAGGCAGGTCGT-3′) was also used to amplify a longer sequence of isotype-1 β-tubulin gene (Niciura et al., 2012).

2.3. Development and optimization of tetra-primer ARMS-PCR system

The annealing temperature was optimized at 60 °C. Different concentration of Mg^{2+} (1.5, 2.0 and 2.5 mM), dNTP (0.15, 0.2 and 0.25 mM) and Taq polymerase (0.5, 1.0 and 1.5U) were used. After optimizing all factors described above, the ratios of the amount of inner primers to that of outer primers (10:1, 5:1, 2:1 and 1:1) were also confirmed.

2.4. Specificity and sensitivity of tetra-primer ARMS-PCR

The specificity of this new assay was determined with three *H. contortus* genomic DNA representing all three known genotypes at codon 198 (homozygous susceptible, heterozygous and homozygous resistant) and distilled water. Meanwhile, the sensitivity of this tetra-primer ARMS-PCR was also checked with serially diluted genomic DNA (10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}) of all three genotypes, which have been measured by Nanodrop 2000 Spectrophotometer.

2.5. Tetra-primer ARMS-PCR for the detection of E198A from field populations

The first PCR was performed from individual genomic DNA in 25 μl reaction system with 1 × buffer, 1.5 mM $MgCl_2$, 0.2 mM each of dNTP, 0.2 μM of each primer F1 and R1, 1 U Taq polymerase (TaKaRa) and 1 μl of gDNA in thermal cycler (Eastwin Life Sciences, Inc., China) under the following protocol: 94 °C/5 min, followed by 35 cycles of 94 °C/55 s, 55 °C/55 s and 72 °C/1 min, with a single final extension cycle of 72 °C for 7 min.

The tetra-primer ARMS-PCR was performed from the first PCR products in 25 μl reaction mixture with 1 × buffer, 2.0 mM $MgCl_2$, 0.2 mM each of dNTP, 0.16 μM of each primer F-outer and R-outer, 0.8 μM of each primer F-inner198s and R-inner198r (the ratio of amount of outer primers to that of inner primers was 1:5), 0.5U Taq polymerase (TaKaRa) and 0.5 μl aliquot of products from the previous reaction with annealing temperature at 60 °C. After counterbalancing the concentration of reaction of tetra-primer ARMS-PCR, all 192 individual *H. contortus* DNA samples from field (Zhang et al., 2016) were subjected to test three genotypes of E198A on 2.0% agarose gels. Finally, three possible outcomes could be detected: samples with amplified PCR products containing two bands of 433 bp and 200 bp in size on agarose gel indicated susceptible homozygous (SS); those with three bands of 433 bp, 284 bp and 200 bp in size indicated heterozygous (RS) and those with two bands of 433 bp and 284 bp in size indicated resistant homozygous (RR). Subsequently, the results of tetra-primer ARMS-PCR were compared with those of PCR-coupled sequencing to evaluate their consistency.

3. Results and discussion

In this study, we have developed a tetra-primer ARMS-PCR method to detect the E198A SNP in the isotype-1 β-tubulin gene of *H. contortus* from eight populations (HuB, HeB, YN, SX, GX, IM, LN and HLJ) in China. We then compared the results of agarose gel electrophoresis with those of direct sequencing from our previous study (Zhang et al., 2016) to test the accuracy of tetra-primer ARMS-PCR.

Three different genomic DNA samples representing three different E198A SNP genotypes (SS, RS and RR) and distilled water were used to test the specificity of tetra-primer ARMS-PCR. Three amplified PCR products containing bands of different sizes could be observed on the agarose gel (Fig. 1). The susceptible homozygous genotype (SS) samples produced two bands with 433 bp and 200 bp in size, respectively. The heterozygous genotype (RS) samples produced three bands with 433 bp, 284 bp and 200 bp in size, respectively. The resistant homozygous genotype (RR) samples produced two bands with 433 bp and 284 bp in size, respectively. Meanwhile, there was no band amplified from distilled water (Fig. 1). The results indicated that there were no non-specific amplification and cross-reactivity in this tetra-primer ARMS-PCR.

The sensitivity of tetra-primer ARMS-PCR was analyzed using serially diluted genomic DNA. For susceptible homozygous genotype (SS), the sensitivity was 4 ng/μL of gDNA (Fig. 2A). For resistant homozygous

Table 1
Primer information and temperatures used in tetra-primer ARMS-PCR including primer names, sequences, their respective melting temperatures and the annealing temperature for reaction.

Primer name	Sequence (5'-3')	Positions change (3' end)	Change	Tm (°C)	Ta (°C)	Size
F-inner198s	ATCAACTGGTAGAGAACACCGACGA	3	T-C	65	60	200 bp
R-inner198r	AGCTTCGTTGTCAATACAGAATGCTG	3	T-C	64		284 bp
F-outer	TCAAAAATTCGTGAAGAGTACCCTGA			64		433 bp
R-outer	ACATTGTGACAGACACTTCAATTGCA			64		