Journal of Helminthology (2017) **91**, 757–761
doi:10.1017/S0022149X16000882

PCR primers for straightforward differentiation of *Haemonchus contortus*, *Haemonchus placei* and their hybrids

M.R.V. Amarante*, M.C. Santos, C.C. Bassetto and A.F.T. Amarante

Departamento de Parasitologia, Instituto de Biociências, UNESP – Universidade Estadual Paulista, Botucatu, SP, Brazil

(Received 27 September 2016; Accepted 2 December 2016; First published online 10 January 2017)

Abstract

Haemonchus contortus and *Haemonchus placei* are among the major parasites of small ruminants and cattle. Although infection with these nematodes is host-specific, with *H. placei* predominating in cattle and *H. contortus* in sheep, cross-infections are observed in areas where both parasites are sympatric, and hybrid offspring can occur. Therefore, a fast and precise method is required for differentiating the parasites. Identification based on spicule morphometry is the most common technique for differentiating *Haemonchus* species. However, because these measurements overlap between species, morphological analysis is insufficient for differentiating between helminth species. In this work, we present a reliable, conventional polymerase chain reaction (PCR)-based method that uses two species-specific primer pairs to differentiate between *H. contortus* and *H. placei* specimens and their hybrids. Each primer pair produces one single and distinct amplification band for each species, which enables the detection of hybrid specimens. These primer pairs were validated by testing eight different populations of *H. contortus*, *H. placei* and hybrids.

Introduction

In tropical and sub-tropical areas of the world, strongyles are the most important group of parasites (Amarante & Amarante, 2016). *Haemonchus contortus* and *Haemonchus placei* are among the major parasites of small ruminants and cattle, respectively. For several decades, *H. placei* was not recognized as a valid species, being considered a synonym of *H. contortus* (Gibbons, 1979). The first evidence that *Haemonchus* from cattle and sheep belonged to different species was reported by Roberts *et al*. (1954) in Australia. They found evidence of some degree of host specificity and concluded that the populations in sheep and cattle represent distinct species: *H. contortus* and *H. placei*, respectively.

In artificial infections, *H. placei* and *H. contortus* establish well in both sheep and cattle hosts (Santos *et al*., 2014; Fávero *et al*., 2016), but host specificity is evident where cattle and sheep share pastures (Santiago *et al*., 1975; Amarante *et al*., 1997). However, cross-infections can occur in areas where both parasites are sympatric, a situation that favours the production of hybrids (Hussain *et al*., 2014). Le Jambre & Royal (1980) demonstrated that hybrid *Haemonchus* produced by crossing *H. placei* and *H. contortus* presented low fecundity or were sterile, due to meiotic abnormalities. Nevertheless, there is recent evidence of genetic flux occurring due to cross-breeding in places where both species are sympatric (Chaudhry *et al*., 2015).

In addition, *H. contortus* developed resistance to all the major classes of anthelmintics, having a significant economic impact on a worldwide scale (Kotze & Prichard, 2016). There also have been reports of anthelmintic resistance in *H. placei*, although the level of resistance is not so extensive (Neves *et al*., 2014). The proper identification of the various species, as well as knowledge regarding the epidemiology of parasitic gastroenteritis, is essential for

*E-mail: monicarv@ibb.unesp.br

the establishment of sustainable strategies of parasite control. Therefore, accurate identification of the two species and their hybrids is imperative in epidemiological studies where the species are sympatric, especially when small ruminants and cattle share the same pastures.

Spicule morphometry is the most frequently used technique for differentiating *Haemonchus* species. However, due to overlapping spicule measurements, a reliable morphometric identification of specimens is not possible (Santos *et al.*, 2014; Vadlejch *et al.*, 2014). It is important to emphasize that the morphological characteristics, despite being useful for specific identification, are based on subtle differences. Therefore, only properly trained personnel are able to perform a precise diagnosis of *H. placei* and *H. contortus* based exclusively on morphometric analysis.

In recent decades, advances in molecular biology have produced several approaches for studying helminth systematics. DNA sequences of the first and second internal transcribed spacers (ITS-1 and ITS-2, respectively) or external transcribed spacer (ETS) of nuclear ribosomal DNA (rDNA) have been used extensively for nematode identification, given their abundance in all genomes (Zarlenga *et al.*, 2001; Von Samson-Himmelstjerna *et al.*, 2002; Gasser *et al.*, 2008; Amarante *et al.*, 2014).

Zarlenga *et al.* (1994) described polymerase chain reaction (PCR) primers 75 and 86, designed from sequences proximal to the 3′- and 5′-ends of the large and small rDNA, that span the ETS sequence. Amplification of *H. contortus* genomic DNA resulted in two DNA fragments of 1.63 and 1.32 kb, while amplification of *H. placei* genomic DNA produced a single 1.63-kb fragment, which enables differentiation between *Haemonchus* sp. However, this primer pair does not detect the presence of hybrids in a population under study because hybrids present the same amplification pattern as parental *H. contortus*.

The aim of the present study was to establish a straightforward, reliable, conventional PCR-based method using species-specific primer pairs to differentiate between *H. contortus*, *H. placei* and hybrid specimens. We designed new sets of primers that amplify exclusively *H. placei* or *H. contortus*, which allow the detection of hybrid specimens. The primer sets were validated with different populations of *H. contortus*, *H. placei* and hybrids.

Materials and methods

DNA isolates

In the present study we used individual male adult specimens of *Haemonchus* of the following isolates, preserved in 70% ethanol.

RsHco1 – laboratory isolate. Adult males were obtained from sheep in a trial described by Santos *et al.* (2014). Originally, this *H. contortus* isolate was obtained from sheep raised in Rio Grande do Sul State, Brazil. It is susceptible to all anthelmintics (Echevarria *et al.*, 1991) and was kindly donated by Dr F.A.M. Echevarria.

SpHco2 – laboratory isolate. Haemonchus contortus isolated from sheep raised in São Paulo State, Brazil with multiple resistance to anthelmintics (Almeida *et al.*, 2010).

MriHco3 – laboratory isolate. Adult specimens of *H. contortus* from Moredun Research Institute, Penicuik, UK. Kindly donated by Dr W. David Smith.

CeHco4 – field isolate. Adult specimens of *H. contortus* obtained from sheep raised in Ceará State, Brazil. Generously donated by Dr Marcel Teixeira (EMBRAPA Caprinos e Ovinos).

SpHco5 – field isolate. Adult specimens of *H. contortus* were obtained from sheep raised in Botucatu, São Paulo State, Brazil by Bassetto *et al.* (2014b).

SpHco6 – field isolate. Adult specimens of *H. contortus* were obtained from sheep raised in Piracicaba, São Paulo State, Brazil by Amarante *et al.* (2009).

SpHpl1 – laboratory isolate. Adult specimens of *H. placei* isolated from cattle raised in São Paulo State, Brazil were obtained by Bassetto *et al.* (2011). The ITS-2 rDNA sequence of this isolate was analysed in another study, confirming *H. placei* identity based on single nucleotide polymorphism (SNP) differences between the *Haemonchus* species (Chaudhry *et al.*, 2015).

SpHpl2 – field isolate. Adult specimens of *H. placei* were obtained from cattle raised in Botucatu, São Paulo State, Brazil by Bassetto *et al.* (2014a).

Hybrids. Hybrids were obtained in our laboratory following an artificial infection of worm-free lambs with 2000 infective larvae (L3) of *H. placei* (SpHpl1 isolate) and 2000 L3 of *H. contortus* (SpHco2 isolate). Faecal samples from these lambs were taken to produce L3 for infecting other worm-free lambs. These last lambs were sacrificed to obtain hybrids (Michelle C. dos Santos, PhD thesis in preparation).

DNA extraction

Genomic DNA was obtained from cattle and sheep blood samples (hosts) and from specimens of *H. contortus* and *H. placei* previously identified based on morphological analysis and used in our laboratory as positive and negative PCR controls. Additionally, DNA was obtained from two hybrids and five biological replicates of each *H. contortus* isolate (RsHco1, SpHco2, MriHco3, CeHco4, SpHco5 and SpHco6) and ten replicates of each *H. placei* isolate (SpHpl1 and SpHpl2). All samples were extracted from single specimens of *Haemonchus* sp. with the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions.

Obtaining new species-specific primer pairs and PCR reactions

Initially, the primer pairs 75 and 86 described by Zarlenga *et al.* (1994) were used to amplify two DNA bands (1.63 and 1.32 kb) from *H. contortus* and a single one (1.63 kb) from *H. placei* DNA. *Haemonchus placei* PCR products were purified using Affymetrix ExoSAP-It (Thermo Fisher, Cleveland, Ohio, USA) and fully sequenced using the Big Dye Terminator V3.1 Cycle sequencing Kit (Applied Biosystems, Foster City, California, USA). Sequencing

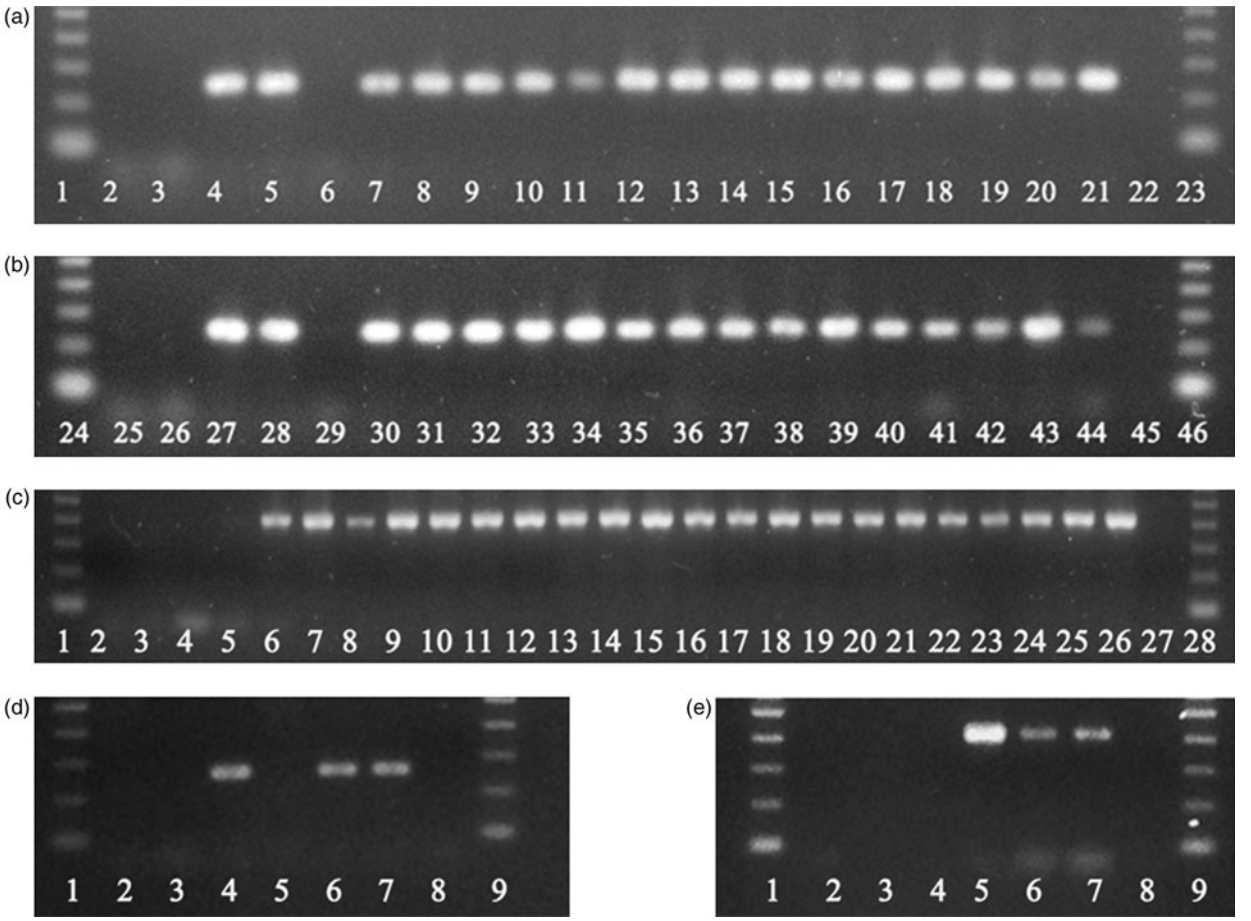

Fig. 1. PCR reactions using DNA samples. (a, b) Species-specific primer pair *HcBotuF1/R2* employed to amplify *H. contortus* from isolates RsHco1 (lanes 7–11), SpHco2 (lanes 12–16), MriHco3 (lanes 17–21), CeHco4 (lanes 30–34), SpHco5 (lanes 35–39) and SpHco6 (lanes 40–44). (c) Species-specific primer pair *HpBotuF/R* employed to amplify *H. placei* from isolates SpHpl1 (lanes 7–16) and SpHpl2 (lanes 17–26). (d and e) **A**mplification with hybrid specimens using species-specific primer pairs: (d) *HcBotuF1/R2* (lanes 6 and 7) and (e) *HpBotuF/R* (lanes 6 and 7). Each photograph also shows reactions with: control samples from bovine (lane 2 in a, c, d and e; lane 25 in b) and ovine hosts (lane 3 in a, c, d and e; lane 26 in b); *H. contortus* (lanes 4 and 5 in a and c; lanes 27 and 28 in b; lane 4 in d and e); *H. placei* (lane 6 in a and c; lane 29 in b; lane 5 in d and e); and reagents without DNA in lanes 22 in a, 45 in b, 27 in c, and 8 in d and e. Molecular markers (100 bp; GE Healthcare) were run in lanes 1 (a, c, d and e), 9 (d and e), 23 (a), 24 (b), 28 (c) and 46 (b).

reactions were analysed on a 3730*xl* DNA Analyzer (Applied Biosystems) and assembled with BioEdit (Hall, 1999). The *H. placei* sequence was compared to known sequences by basic local alignment tool (BLAST) analysis against the National Center for Biotechnology Information (NCBI) database, in order to identify the rDNA regions. The 1.63-kb *H. placei* sequence was also compared to HQ389234.1 (*H. contortus*) sequence (NCBI database) by BLAST analysis.

Then, two species-specific primer pairs were designed using Primer3 (Rozen & Skaletsky, 2000): for *H. placei*, *HpBotuF* 5′-CCAGACCCGAGACTCGCC-3′ and *HpBotuR* 5′-CTGAAGGTAATGTCAAAATTTCT-3′; for *H. contortus*, *HcBotuF1* 5′-TGTCGAACACGAAACTCGTC-3′ and *Hc BotuR2* 5′-TGTGTCTCTACCGCCCGAGT-3′.

Each DNA sample was tested with both primer pairs (*HpBotuF/R* and *HcBotuF1/R2*). The Gene Amp PCR System 9700® thermocycler (Applied Biosystems) was employed to perform PCR reactions. Amplification was carried out in a 10 µl reaction mix containing 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl (pH 8.0), 100 µM each deoxynucleoside triphosphate (dNTP), 20–50 ng genomic DNA and 0.5 U *Taq* polymerase (Invitrogen, Carlsbad, California, USA). The cycling conditions for the primer pair *HpBotuF/R* were as follows: 95°C for 5 min; followed by 35 cycles of 95°C for 30 s, 58.5°C for 15 s and 72°C for 30 s; followed by 5 min at 72°C and 4°C to finalize. The cycling conditions for the primer pair *HcBotuF1/R2* were largely the same, except the annealing time and temperature: 59°C for 40 s. PCR products were electrophoresed on 2% agarose gels in 1% Tris–acetate–EDTA (TAE) buffer containing ethidium bromide, and photographed under UV light using a Sony Cyber-shot DSC-HX1 camera (Sony Electronics, San Diego, California, USA).

Results and discussion

PCR amplification (fig. 1) was used to verify primer pair specificity by using control DNA samples from

bovine (lane 2 in a, c, d and e; lane 25 in b) and ovine hosts (lane 3 in a, c, d and e; lane 26 in b), *H. contortus* (lanes 4, 5 in a and c; lanes 27 and 28 in b; lane 4 in d and e) and *H. placei* (lane 6 in a and c; lane 29 in b; lane 5 in d and e).

An amplification band was obtained with all *H. contortus* samples with the *HcBotuF1/R2* primer pair (fig. 1a and b), while no amplification of these samples was observed with the *HpBotuF/R* primer pair. The opposite occurred with DNA samples from *H. placei* (fig. 1c).

Figure 1a shows the result of the use of *HcBotuF1/R2* primer pair. Lanes 7–11 and 12–16, which are from laboratory isolates RsHco1 and SpHco2 (from *H. contortus* – Rio Grande do Sul and São Paulo, Brazil) present a 260-bp amplification product. Lanes 17–21 also show a 260-bp band in MriHco3 samples, an *H. contortus* laboratory isolate (the Moredun Research Institute, UK). Additionally, a 260-bp PCR band is presented in fig. 1b, lanes 30–34, 35–39 and 40–44, which are from the CeHco4, SpHco5 and SpHco6 Brazilian field isolates, one isolated in Ceará State and two in São Paulo State, respectively. Lanes 4, 5, 27 and 28 are positive *H. contortus* controls, and no amplification band was observed in lanes 6 and 29 (*H. placei* DNA control samples).

In fig. 1c, the species-specific primer pair *HpBotuF/R* was employed, and a 459-bp amplification band is presented in all *H. placei* samples. Lane 6 is a positive control, and no amplification band was observed in lanes 4 and 5 (*H. contortus* DNA control samples). A 459-bp PCR band was observed in lanes 7–16 and lanes to 17–26, which are SpHpl1 and SpHpl2 laboratory isolates, respectively, from São Paulo state, Brazil.

PCR of two hybrid samples using the species-specific primer pairs *HcBotuF1/R2* (fig. 1d) and *HpBotuF/R* (fig. 1e) was performed with DNA samples from hosts (bovine and ovine, lanes 2 and 3, respectively), control samples (*H. contortus* and *H. placei*, lanes 4 and 5, respectively) and hybrids (lanes 6 and 7). The presence of hybrids is evident because a 260-bp amplification band (from *H. contortus*) is observed in lanes 6 and 7 of fig. 1d and a 459-bp band (from *H. placei*) is presented in lanes 6 and 7 of fig. 1e.

In Brazil, cattle and sheep usually share pastures, and a specific diagnosis is important because these ruminants normally harbour mixed infections, favouring the production of hybrids (Hussain *et al.*, 2014). This phenomenon can result in genetic flux between populations, with implications for interspecies transmission of resistance genes (Chaudhry *et al.*, 2015).

Therefore, a precise identification of *Haemonchus* species is essential for differentiating between these species and their hybrids. PCR primer pairs have been employed successfully to identify nematode genera (Schnieder *et al.*, 1999; Von Samson-Himmelstjerna *et al.*, 2002). In this work, we present a reliable conventional PCR-based method with just two species-specific primer pairs that can be used to identify individual specimens of *H. contortus* and *H. placei* and their hybrids.

It is important to mention that the *HcBotuF1/R2* primer pair was used to identify laboratory (RsHco1, SpHco2 and MriHco3) and field isolates (CeHco4, SpHco5 and SpHco6) of *H. contortus* from different places. RsHco1, SpHco2 isolates are kept in Brazilian laboratories (Echevarria *et al.*, 1991; Almeida *et al.*, 2010) and MriHco3 in the Moredun Research Institute, UK. The other three *H. contortus* field isolates are originally from two distinct places in Brazil, São Paulo (Amarante *et al.*, 2009; Bassetto *et al.*, 2014b) and Ceará states, which are located in the south-eastern and north-eastern areas of Brazil, respectively. It is important to stress that these states present very different weather, temperature and precipitation ranges throughout the year, which corroborates our assertion that the *HcBotuF1/R2* primer pair is able to identify positively a spectrum of *H. contortus* DNA samples.

In conclusion, testing the two primer pairs (*HpBotuF/R* and *HcBotuF1/R2*) in eight different populations of *H. contortus* and *H. placei* has demonstrated the primers' accuracy in the differentiation of individual and hybrid species.

Financial support

This study was supported by the São Paulo Research Foundation (FAPESP, grant number 2014/02305-6). M.C.S. and C.C.B. received financial support from FAPESP (grant numbers 2012/23941-2 and 2015/00221-2, respectively). A.F.T.A. received support from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, grant number 3037125/2013-9).

Conflict of interest

None.

Ethical standards

This trial was approved and conducted in accordance with the experimental protocol approved by the local Ethical Committee (protocol number 449-CEEA).

References

Almeida, F.A., Garcia, K.C.O.D., Torgerson, P.R. & Amarante, A.F.T. (2010) Multiple resistance to anthelmintics by *Haemonchus contortus* and *Trichostrongylus colubriformis* in sheep in Brazil. *Parasitology International* **59**, 622–625.

Amarante, A.F.T. & Amarante, M.R.V. (2016) Advances in the diagnosis of the gastrointestinal nematode infections in ruminants. *Brazilian Journal of Veterinary Research and Animal Science* **53**, 127–137.

Amarante, A.F.T., Bagnola, J. Jr, Amarante, M.R.V. & Barbosa, M.A. (1997) Host specificity of sheep and cattle nematodes in São Paulo state, Brazil. *Veterinary Parasitology* **73**, 89–104.

Amarante, A.F.T., Susin, I., Rocha, R.A., Silva, M.B., Mendes, C.Q. & Pires, A.V. (2009) Resistance of Santa Ines and crossbred ewes to naturally acquired gastrointestinal nematode infections. *Veterinary Parasitology* **165**, 273–280.

Amarante, M.R.V., Bassetto, C.C., Neves, J.H. & Amarante, A.F.T. (2014) Species-specific PCR for the identification of *Cooperia curticei* (Nematoda: Trichostrongylidae) in sheep. *Journal of Helminthology* **88**, 447–452.

Bassetto, C.C., Silva, B.F., Newlands, G.F.J., Smith, W.D. & Amarante, A.F.T. (2011) Protection of calves against *Haemonchus placei* and *Haemonchus contortus* after immunization with gut membrane proteins from *H. contortus*. *Parasite Immunology* **33**, 377–381.

Bassetto, C.C., Silva, M.R.L., Newlands, G.F.J., Smith, W. D., Ratti Júnior, J., Martins, C.L. & Amarante, A.F.T. (2014a) Vaccination of grazing calves with antigens from the intestinal membranes of *Haemonchus contortus*: effects against natural challenge with *Haemonchus placei* and *Haemonchus similis*. *International Journal for Parasitology* **44**, 697–702.

Bassetto, C.C., Picharillo, M.E., Newlands, G.F.J., Smith, W.D., Fernandes, S., Siqueira, E.R. & Amarante, A.F. T. (2014b) Attempts to vaccinate ewes and their lambs against natural infection with *Haemonchus contortus* in a tropical environment. *International Journal for Parasitology* **44**, 1049–1054.

Chaudhry, U., Redman, E.M., Abbas, M., Muthusamy, R., Ashraf, K. & Gilleard, J.S. (2015) Genetic evidence for hybridisation between *Haemonchus contortus* and *Haemonchus placei* in natural field populations and its implications for interspecies transmission of anthelmintic resistance. *International Journal for Parasitology* **45**, 149–159.

Echevarria, F.A.M., Armour, J. & Duncan, J.L. (1991) Efficacy of some anthelmintics on a ivermectin-resistant strain of *Haemonchus contortus* in sheep. *Veterinary Parasitology* **39**, 279–284.

Fávero, F.C., Buzzulini, C., Cruz, B.C., Felippelli, G., Maciel, W.G., Salatta, B., Teixeira, W.F.P., Soares, V. E., Oliveira, G.P., Lopes, W.D.Z. & Costa, A.J. (2016) Experimental infection of calves with *Haemonchus placei* and *Haemonchus contortus*: assessment of parasitological parameters. *Veterinary Parasitology* **217**, 25–28.

Gasser, R.B., Bott, N.J., Chilton, N.B., Hunt, P. & Beveridge, I. (2008) Toward practical, DNA-based diagnostic methods for parasitic nematodes of livestock – bionomic and biotechnological implications. *Biotechnology Advances* **26**, 325–334.

Gibbons, L.M. (1979) Revision of the genus *Haemonchus* Cobb, 1898 (Nematoda: Trichostrongilidae). *Systematic Parasitology* **1**, 3–24.

Hall, TA. (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program. *Nucleic Acids Symposium Series* **41**, 95–98.

Hussain, T., Periasamy, K., Nadeem, A., Babar, M.E., Pichler, R. & Diallo, A. (2014) Sympatric species distribution, genetic diversity and population structure of *Haemonchus* isolates from domestic ruminants in Pakistan. *Veterinary Parasitology* **206**, 188–199.

Kotze, A.C. & Prichard, R.K. (2016) Anthelmintic resistance in *Haemonchus contortus*: history, mechanisms and diagnosis. pp. 397–428 *in* Gasser, R.B. & Von Sampson-Himmelstjerna, G. (*Eds*) Haemonchus contortus *and haemonchosis – past, present and future trends. Advances in Parasitology*, vol. **93**. Amsterdam, Elsevier.

Le Jambre, L.F. & Royal, W.M. (1980) Meiotic abnormalities in backcross lines of hybrid *Haemonchus*. *International Journal for Parasitology* **10**, 281–286.

Neves, J.H., Carvalho, N., Rinaldi, L., Cringoli, G. & Amarante, A.F.T. (2014) Diagnosis of anthelmintic resistance in cattle in Brazil: a comparison of different methodologies. *Veterinary Parasitology* **206**, 216–226.

Roberts, F.H.S., Newton-Turner, H. & McKevett, M. (1954) On the specific distinctness of the ovine and bovine 'Strains' of *Haemonchus contortus* (Rudolphi) Cobb (Nematoda: Trichostronglidae). *Australian Journal of Zoology* **2**, 275–295.

Rozen, S. & Skaletsky, H.J. (2000) Primer3 on the WWW for general users and for biologist programmers. pp. 365–386 *in* Krawetz, S. & Misener, S. (*Eds*) *Bioinformatics methods and protocols: Methods in molecular biology*. Totowa, NJ, Humana Press. Source code available at http://fokker.wi.mit.edu/primer3/ (accessed 1 August 2015).

Santiago, M.A.M., Costa, U.C. & Benevenga, S.F. (1975) Estudo comparativo da prevalência de helmintos em ovinos e bovinos criados na mesma pastagem. *Pesquisa Agropecuária Brasileira, Série Veterinária* **10**, 51–58.

Santos, M.C., Xavier, J.K., Amarante, M.R.V., Bassetto, C.C. & Amarante, A.F.T. (2014) Immune response to *Haemonchus contortus* and *Haemonchus placei* in sheep and its role in parasite specificity. *Veterinary Parasitology* **203**, 127–138.

Schnieder, T., Heise, M. & Epe, C. (1999) Genus-specific PCR for the differentiation of eggs or larvae from gastrointestinal nematodes of ruminants. *Parasitology Research* **85**, 895–898.

Vadlejch, J., Lukešová, D., Vašek, J., Vejl, P., Sedlák, P., Čadková, Z., Langrova, I., Jankovská, I. & Salaba, O. (2014) Comparative morphological and molecular identification of *Haemonchus* species in sheep. *Helminthologia* **51**, 130–140.

Von Samson-Himmelstjerna, G.A., Harder, A. & Schnieder, T. (2002) Quantitative analysis of ITS2 sequences in trichostrongyle parasites. *International Journal for Parasitology* **32**, 1529–1535.

Zarlenga, D.S, Stringfellow, F., Nobary, M. & Lichtenfels, J.R. (1994) Cloning and characterization of ribosomal RNA genes from three species of *Haemonchus* (Nematoda: Trichostrongyloidea) and identification of PCR primers for rapid differentiation. *Experimental Parasitology* **78**, 28–36.

Zarlenga, D.S., Chute, M.B., Gasbarre, L.C. & Boyd, P.C. (2001) A multiplex PCR assay for differentiating economically important gastrointestinal nematodes of cattle. *Veterinary Parasitology* **97**, 201–211.