Journal of Helminthology (2015) **89,** 118–123
doi:10.1017/S0022149X13000308

Molecular differentiation of *Trichinella spiralis, T. pseudospiralis, T. papuae* and *T. zimbabwensis* by pyrosequencing

L. Sadaow[1,2], C. Tantrawatpan[2,3], P.M. Intapan[1,2], V. Lulitanond[2,4], T. Boonmars[1], N. Morakote[5], E. Pozio[6] and W. Maleewong[1,2]*

[1]Department of Parasitology, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand: [2]Research and Diagnostic Center for Emerging Infectious Diseases, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand: [3]Division of Cell Biology, Department of Preclinical Sciences, Faculty of Medicine, Thammasat University, Rangsit Campus, Pathum Thani 12121, Thailand: [4]Department of Microbiology, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand: [5]Department of Parasitology, Faculty of Medicine, Chiang Mai University, Chiang Mai 50200, Thailand: [6]Department of Infectious, Parasitic and Immunomediated Diseases, Istituto Superiore di Sanità, viale Regina Elena 299, 00161 Rome, Italy

(Received 16 January 2013; Accepted 2 April 2013; First Published Online 13 May 2013)

Abstract

Nematodes of the genus *Trichinella* which infect wildlife and domestic animals show a cosmopolitan distribution. These zoonotic parasites are the aetiological agents of a severe human disease, trichinellosis. Twelve taxa are recognized in the *Trichinella* genus, but they cannot be identified by morphology since they are sibling species/genotypes. For epidemiological studies, it is extremely important to identify each taxon since they have different distribution areas and host ranges. In the present study, polymerase chain reaction (PCR) amplification of the mitochondrial large subunit ribosomal RNA (lsu-RNA) gene coupled with a pyrosequencing technique was developed to distinguish among four *Trichinella* species: *Trichinella spiralis*, *T. pseudospiralis*, *T. papuae* and *T. zimbabwensis*. A PCR method was used to amplify the lsu-RNA of *Trichinella* sp. larvae in mouse muscles and single larvae collected from infected muscles by digestion. The results show that the four *Trichinella* species can be distinguished by using 26 nucleotides in the target region and the method is sensitive enough to identify individual larvae. The pyrosequencing provides a simple, rapid and high-throughput tool for the differentiation of *Trichinella* species.

Introduction

Trichinellosis is a cosmopolitan zoonosis caused by the ingestion of raw meat infected by roundworms of the genus *Trichinella*. Currently, the *Trichinella* genus consists of two clades: the non-encapsulated clade with three species infecting mammals and birds (*T. pseudospiralis*), or mammals and reptiles (*T. papuae* and *T. zimbabwensis*), and the encapsulated clade with six species (*Trichinella spiralis*, *T. nativa*, *T. britovi*, *T. murrelli*, *T. nelsoni* and *T. patagoniensis*) and three genotypes (*Trichinella* T6, T8 and T9) infecting only mammals (Pozio *et al.*, 2009;

*E-mail: wanch_ma@kku.ac.th

Krivokapich *et al.*, 2012). The differentiation of *Trichinella* taxa is of importance for biological and epidemiological studies. The tools for the identification of *Trichinella* species were first based on biological (e.g. host specificity, geographical distribution, cross-breeding, nurse cell development and resistance to freezing), biochemical (e.g. isoenzyme analysis) and molecular methods (Murrell *et al.*, 2000). A plethora of molecular diagnostic methods based on the conventional polymerase chain reaction (PCR) (Dick *et al.*, 1992; Bandi *et al.*, 1995), PCR-restriction fragment length polymorphism (RFLP) (Wu *et al.*, 1999), PCR-based single strand conformation polymorphism analysis (Gasser *et al.*, 1998), multiplex PCR (Zarlenga *et al.*, 1999) and real-time PCR (Guenther *et al.*, 2008; Tantrawatpan *et al.*, 2012) are available to distinguish *Trichinella* taxa.

Recently, the pyrosequencing technique has facilitated direct sequencing by the synthesis of short nucleotide fragments using an enzymatic-cascade system (Ahmadian *et al.*, 2006). This method has been used for high-throughput genotyping of protozoan parasites (Sreekumar *et al.*, 2005; Stensvold *et al.*, 2007, 2010; Lulitanond *et al.*, 2012) and allows the detection of nucleotide polymorphisms that can be used for parasite differentiation at the species level. The aim of the present work was to develop a tool to identify, by PCR and pyrosequencing, four *Trichinella* species, three of which are circulating in humans and animals of South-East Asia.

Materials and methods

Sources of Trichinella *larvae*

Trichinella sp. larvae belonging to four species, i.e. *T. spiralis*, *T. papuae*, *T. pseudospiralis* and *T. zimbabwensis*, were used in this study. The *T. spiralis* strain (code ISS62), maintained in laboratory mice by serial passages, originated from a domestic pig that was the source of infection for a human outbreak in the Mae Hong Son Province, Thailand, in 1986 (Pozio & Khamboonruang, 1989). The *T. pseudospiralis* strain (code ISS13) was the original strain isolated from Russia in 1972 (Garkavi, 1972). The *T. zimbabwensis* (code ISS1029) strain was isolated from a Nile crocodile of Zimbabwe in 1996 (Pozio *et al.*, 2002). The *T. papuae* (code ISS4120) strain was isolated from a muscle biopsy of a patient who had worked in Malaysia and had a history of eating raw pork from a wild boar in 2005 (Intapan *et al.*, 2011). *Trichinella* larvae were isolated from mouse muscles by digestion and stored in 70% ethanol at −20°C. This study was approved by the Animal Ethics Committee of the Khon Kaen University, based on the Ethics of Animal Experimentation of the National Research Council of Thailand (Reference No. 0514.1.12.2/70).

Molecular analysis

The mitochondrial large subunit ribosomal RNA (lsu-RNA) of *T. spiralis* (GU339148), *T. papuae* (AY851286), *T. pseudospiralis* (DQ159091) and *T. zimbabwensis* (EF5-17131), available in GenBank were selected to find a suitable region to distinguish the four species. *Trichinella* genus specific PCR primers (TriPyr_F, 5′-TAGATTGTGA-CCTCGATGTTGAA-3′ and biotinylated TriPyr_R, biotin-5′-AAAGAGAATCCAACCTGTCTTGC-3′) and sequencing primer (TriPyr_S, 5′-CCTCGATGTTGAATCA-3′) were designed by using pyrosequencing assay design software (PyroMark™ Q96 ID software version 1.0; Biotage, Uppsala, Sweden) (fig. 1).

Muscle larvae were isolated from mouse muscles by an artificial pepsin digestion (Nöckler & Kapel, 2007). Ten larvae of each species mixed with 250 mg of mouse muscles (equivalent to approximately 40 larvae/g), and each species of *Trichinella* larvae (one or ten larvae), were homogenized with a disposable polypropylene pestle, followed by DNA extraction using a NucleoSpin Tissue kit (Macherey-Nagel GmbH & Co., Duren, Germany) according to the manufacturer's instructions. The DNA was eluted in 50 µ of distilled water, and 5 µl of this solution was used for PCR. The PCR products were obtained from conventional PCRs using the TriPyr_F and TriPyr_R primers. Positive control plasmids containing *T. spiralis*, *T. papuae*, *T. pseudospiralis* and *T. zimbabwensis* DNA were constructed by cloning the 127 bp PCR product of the lsu-RNA into the pGEM®-T Easy vector (Promega, Madison, Wisconsin, USA) according to the manufacturer's instructions. The recombinant plasmids were propagated in *Escherichia coli*, and the cloned inserts were sequenced to confirm their identity.

Fig. 1. Alignment of the mitochondrial large subunit ribosomal RNA gene of *Trichinella spiralis* (GU339148), *T. papuae* (AY851286), *T. pseudospiralis* (DQ159091) and *T. zimbabwensis* (EF517131), showing the position of the forward and reverse PCR primers (arrows), of the sequencing primer (solid rectangle) and the position of target regions used for the species differentiation (dotted rectangle).

https://doi.org/10.1017/S0022149X13000308 Published online by Cambridge University Press

The 127 bp of the lsu-RNA were amplified from genomic DNAs of larvae of the four *Trichinella* species, of mouse muscle samples spiked with *Trichinella* larvae, and of positive control plasmids, on a GeneAmp PCR system 9700 thermal cycler (Applied Biosystems, Singapore). The PCR mix consisted of 1 × PCR buffer (Invitrogen, Carlsbad, California, USA) with 0.2 mM of each deoxyribonucleoside triphosphate (dNTP), 1.5 mM $MgSO_4$, 0.2 μM each of TriPyr_F primer and biotinylated TriPyr_R primer, 0.625 U of Platinum *Taq* DNA polymerase

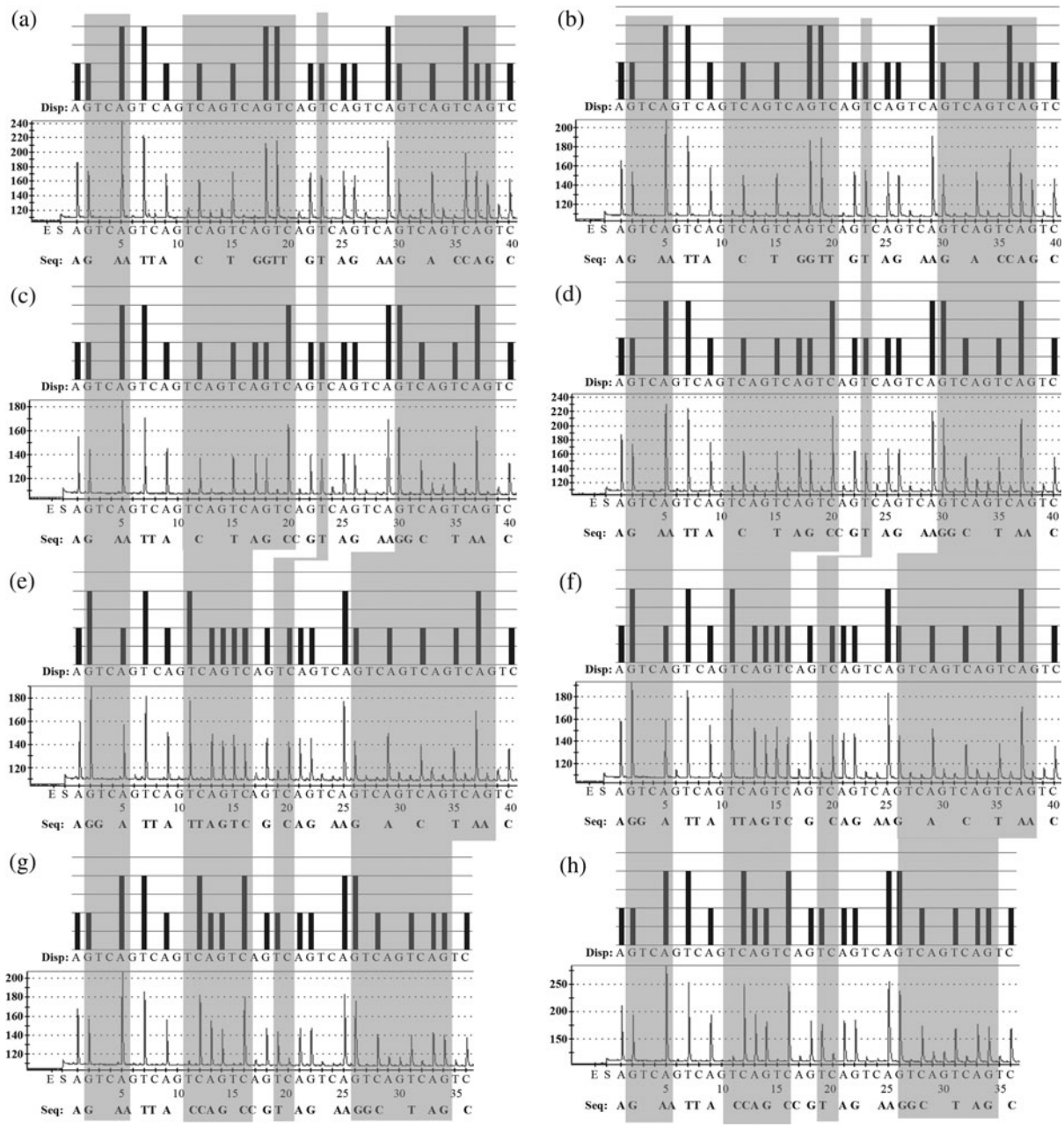

Fig. 2. Pyrograms showing the sequence analyses of *Trichinella spiralis* (a, b), *T. papuae* (c, d), *T. pseudospiralis* (e, f) and *T. zimbabwensis* (g, h) genes. The pyrogram patterns (top of each panel), representative raw data (bottom of each panel) of control plasmids (a, c, e, g), and DNA extracted from each larva of the four *Trichinella* species spiked in mouse muscles (b, d, f, h), are shown. The letters under the black bars show the dispensation (Disp) order. The sequence (Seq) detected by pyrosequencing is shown below each panel. The *y*-axis shows the fluorescence emission by the incorporation of a nucleotide base; the *x*-axis shows the bases added at that point in time of the pyrosequencing. The light grey areas show the pyrogram for the differentiation of each of the four target *Trichinella* species. E, enzyme; S, substrate; A, G, T, C, four different nucleotides.

https://doi.org/10.1017/S0022149X13000308 Published online by Cambridge University Press

high fidelity (Invitrogen) and 5 µl of genomic DNA, in a final reaction volume of 25 µl. The amplification procedure was as follows: 5 min at 94°C for initial denaturation followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 30 s, followed by a final extension at 72°C for 7 min. Electrophoresis on a 1.5% agarose gel was performed to verify the amplification of single products. For the analytical specificity, DNA samples of organisms other than those of the genus *Trichinella* (helminths: *Ascaris lumbricoides*, *Ancylostoma caninum*, *Trichuris trichiura*, *Capillaria philippinensis*, *Strongyloides stercoralis*, *Trichostrongylus* spp., *Taenia* spp., *Opisthorchis viverrini*, *Haplorchis taichui*, *Clonorchis sinensis*, *Centrocestus* spp., *Stellantchasmus* spp., *Paragonimus heterotremus*, *Schistosoma mekongi*, *Fasciola gigantica*, *Echinostoma malayanum* and *Phaneropsorus boneii*; protozoa: *Giardia lamblia* and *Isospora belli*; human and mouse) were tested.

Following PCR amplification, biotinylated PCR products were placed in 96-well plates and bound to Streptavidin Sepharose™ beads (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The PCR products immobilized with beads were denatured and the non-biotinylated fragments were washed from the beads using a PyroMark™ Vacuum Prep Workstation (Biotage). Subsequently, the beads were released and resuspended in 40 µl annealing buffer (20 mM Tris-acetate, 5 mM magnesium acetate, pH 7.6) containing 0.4 µM TriPyr_S sequencing primer. Samples in duplicate were heated at 80°C for 2 min before performing the pyrosequencing reaction using PyroMark™ Gold Q96 SQA reagents (Biotage) on a PSQ™ 96MA instrument (Biotage). For each assay, two negative control samples were included (sequencing primer and biotinylated primer, only). Following the completion of the pyrosequencing reaction, the PyroMark™ Q96 ID software version 1.0 (Biotage) was run to produce a pyrogram and to analyse the sequencing data. The readouts were interpreted manually in cases where the target sequences contained up to four homopolymers, because of software limitations.

Results and discussion

No amplification products were obtained with DNA samples of organisms different from those of the genus *Trichinella* (see the Materials and methods section for a list). The DNA pyrosequencing of 26 nucleotides of the target region (nucleotide number ranging from 2019 to 2044) (fig. 1) unequivocally identified each of the four *Trichinella* species (fig. 2). The nucleotides present at positions 2021, 2026–2028, 2030–2031, 2033, 2039, 2041 and 2043, were used for the species identification. For *T. spiralis*, specific nucleotides were found at positions 2028 (G), 2031 (T) and 2041 (C). For the non-encapsulated clade, specific nucleotides were found at positions 2028 (A), 2031 (C) and 2041 (T) (fig. 2 and table 1). The species *T. papuae* and *T. zimbabwensis* were distinguished at positions 2030 (C) and 2039 (G). In the case of *T. pseudospiralis*, specific nucleotides were found at positions 2021 (G), 2026 (T) and 2033 (C). Finally, in the case of *T. zimbabwensis*, the presence of a C at position 2027 allows its identification (fig. 2 and table 1). No difference was observed for each species among control plasmids, DNA extracted from one or ten larvae, and DNA extracted from mouse muscles spiked with ten *Trichinella* larvae. DNA sequences from pyrosequencing of each sample were identical to the sequences produced by Sanger sequencing. The negative controls did not yield pyrograms.

Pyrosequencing is becoming more common for the rapid differentiation and single nucleotide genotyping of protozoan parasites such as *Toxoplasma gondii* (Sreekumar *et al.*, 2005), *Blastocystis hominis* (Stensvold *et al.*, 2007), *Entamoeba* complex (Stensvold *et al.*, 2010), and *Plasmodium vivax* and *P. falciparum* (Lulitanond *et al.*, 2012). Laboratory personnel can be easily trained to perform this technique because the pyrosequencing procedure is relatively simple. Furthermore, the cost of pyrosequencing reagents is lower than that of conventional sequencing reagents. After the PCR amplification, the pyrosequencing run time for 96 samples is approximately 1 h.

To the best of our knowledge, this is the first work to identify *Trichinella* larvae at the species level by pyrosequencing. DNA pyrosequencing coupled with PCR amplification, using a new primer set targeting the highly conserved region of the lsu-RNA sequence, yields species-level differentiation of the three non-encapsulated *Trichinella* species and the encapsulated *T. spiralis*, three of which are circulating in South-East Asia (Jongwutiwes *et al.*, 1998; Pozio *et al.*, 2009; Kusolsuk *et al.*, 2010; Intapan *et al.*, 2011; Van De *et al.*, 2012). This new diagnostic tool shows high sensitivity and specificity. In fact, it allows identification of single *Trichinella* larvae, and no amplification product was detected with DNAs from other parasites, either helminths or protozoa.

Table 1. Regions of the mitochondrial large subunit ribosomal RNA (lsu-RNA) gene and nucleotide patterns used for differentiation of *Trichinella spiralis*, *T. pseudospiralis*, *T. papuae* and *T. zimbabwensis* by pyrosequencing; GenBank numbers for *T. spiralis* (GU339148), *T. papuae* (AY851286), *T. pseudospiralis* (DQ159091) and *T. zimbabwensis* (EF517131).

	Nucleotide positions									
Trichinella species	2021	2026	2027	2028	2030	2031	2033	2039	2041	2043
T. spiralis	A	C	T	G[e]	T	T[e]	T	A	C[e]	G
T. papuae	A	C	T	A[a]	C[d]	C[a]	T	G[d]	T[a]	A
T. pseudospiralis	G[b]	T[b]	T	A[a]	T	C[a]	C[b]	A	T[a]	A
T. zimbabwensis	A	C	C[c]	A[a]	C[d]	C[a]	T	G[d]	T[a]	G

[a]Specific nucleotide for non-encapsulated *Trichinella* species, [b]*T. pseudospiralis*, [c]*T. zimbabwensis*, [d]*T. papuae* and *T. zimbabwensis*, and [e]*T. spiralis*.

https://doi.org/10.1017/S0022149X13000308 Published online by Cambridge University Press

In this study, only one isolate has been analysed for each of the four *Trichinella* species. Therefore, nucleotide variation in the target region needs to be investigated among isolates of these species from different geographical origins. For the bioinformatic analysis, the comparison with the other *Trichinella* taxa needs further investigations, since the 26-nucleotide sequence of *T. spiralis* is identical to that of other encapsulated taxa (data not shown). This rapid and specific assay is a promising alternative method that can be used for the differentiation of larvae of *Trichinella* taxa present in muscle tissues of both domestic and wild animals.

Acknowledgements

This research was funded by grants from the National Science and Technology Development Agency (Discovery Based Development Grant); the Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission, Thailand; the Faculty of Medicine, Khon Kaen University. L.S. was supported by the Khon Kaen University grant. W.M. was supported by TRF Senior Research Scholar Grant, Thailand Research Fund grant number RTA5580004. This work was also partially supported by the Directorate-General for Health and Consumers (DG SANCO) of the European Commission (2011 funds).

References

Ahmadian, A., Ehn, M. & Hober, S. (2006) Pyrosequencing: history, biochemistry and future. *Clinica Chimica Acta* **363**, 83–94.

Bandi, C., La Rosa, G., Bardin, M.G., Damiani, G., Comincini, S., Tasciotti, L. & Pozio, E. (1995) Random amplified polymorphic DNA fingerprints of the eight taxa of *Trichinella* and their comparison with allozyme analysis. *Parasitology* **110**, 401–407.

Dick, T.A., Lu, M.C., deVos, T. & Ma, K. (1992) The use of the polymerase chain reaction to identify porcine isolates of *Trichinella*. *Journal of Parasitology* **78**, 145–148.

Garkavi, B.L. (1972) The species of *Trichinella* isolated from wild carnivores. *Veterinariia* **10**, 90–91.

Gasser, R.B., Zhu, X.Q., Monti, J.R., Dou, L., Cai, X. & Pozio, E. (1998) PCR-SSCP of rDNA for the identification of *Trichinella* isolates from mainland China. *Molecular and Cellular Probes* **12**, 27–34.

Guenther, S., Nockler, K., von Nickisch-Rosenegk, M., Landgraf, M., Ewers, C., Wieler, L.H. & Schierack, P. (2008) Detection of *Trichinella spiralis*, *T. britovi* and *T. pseudospiralis* in muscle tissue with real-time PCR. *Journal of Microbiological Methods* **75**, 287–292.

Intapan, P.M., Chotmongkol, V., Tantrawatpan, C., Sanpool, O., Morakote, N. & Maleewong, W. (2011) Molecular identification of *Trichinella papuae* from a Thai patient with imported trichinellosis. *American Journal of Tropical Medicine and Hygiene* **84**, 994–997.

Jongwutiwes, S., Chantachum, N., Kraivichian, P., Siriyasatien, P., Putaporntip, C., Tamburrini, A., La Rosa, G., Sreesunpasirikul, C., Yingyourd, P. & Pozio, E. (1998) First outbreak of human trichinellosis caused by *Trichinella pseudospiralis*. *Clinical Infectious Diseases* **26**, 111–115.

Krivokapich, S.J., Pozio, E., Gatti, G.M., Prous, C.L., Ribicich, M., Marucci, G., La Rosa, G. & Confalonieri, V. (2012) *Trichinella patagoniensis* n. sp. (Nematoda), a new encapsulated species infecting carnivorous mammals in South America. *International Journal for Parasitology* **42**, 903–910.

Kusolsuk, T., Kamonrattanakun, S., Wesanonthawech, A., Dekumyoy, P., Thaenkham, U., Yoonuan, T., Nuamtanong, S., Sa-Nguankiat, S., Pubampen, S., Maipanich, W., Panitchakit, J., Marucci, G., Pozio, E. & Waikagul, J. (2010) The second outbreak of trichinellosis caused by *Trichinella papuae* in Thailand. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **104**, 433–437.

Lulitanond, V., Intapan, P.M., Tantrawatpan, C., Sankuntaw, N., Sanpool, O., Janwan, P. & Maleewong, W. (2012) Molecular markers for detection and differentiation of *Plasmodium falciparum* and *Plasmodium vivax* in human blood samples by pyrosequencing. *Journal of Clinical Microbiology* **50**, 1455–1457.

Murrell, K.D., Lichtenfels, R.J., Zarlenga, D.S. & Pozio, E. (2000) The systematics of the genus *Trichinella* with a key to species. *Veterinary Parasitology* **93**, 293–307.

Nöckler, K. & Kapel, C.M.O. (2007) Chapter 3. Detection and surveillance for *Trichinella*: meat inspection and hygiene, and legislation. pp. 69–97 *in* Dupouy-Camet, J. & Murrell, K.D. (*Eds*) *FAO/WHO/OIE Guidelines for the surveillance, management, prevention and control of trichinellosis*. Paris, World Organisation for Animal Health (OIE).

Pozio, E. & Khamboonruang, C. (1989) Trichinellosis in Thailand: epidemiology and biochemical identification of the aethiological agent. *Tropical Medicine and Parasitology* **40**, 73–74.

Pozio, E., Foggin, C.M., Marucci, G., La Rosa, G., Sacchi, L., Corona, S., Rossi, P. & Mukaratirwa, S. (2002) *Trichinella zimbabwensis* n.sp. (Nematoda), a new non-encapsulated species from crocodiles (*Crocodylus niloticus*) in Zimbabwe also infecting mammals. *International Journal for Parasitology* **32**, 1787–1799.

Pozio, E., Hoberg, E., La Rosa, G. & Zarlenga, D.S. (2009) Molecular taxonomy, phylogeny and biogeography of nematodes belonging to the *Trichinella* genus. *Infection, Genetics and Evolution* **9**, 606–616.

Sreekumar, C., Hill, D.E., Miska, K.B., Vianna, M.C., Yan, L., Myers, R.L. & Dubey, J.P. (2005) Genotyping and detection of multiple infections of *Toxoplasma gondii* using pyrosequencing. *International Journal for Parasitology* **35**, 991–999.

Stensvold, C.R., Traub, R.J., von Samson-Himmelstjerna, G., Jespersgaard, C., Nielsen, H.V. & Thompson, R.C. (2007) *Blastocystis*: subtyping isolates using pyrosequencing technology. *Experimental Parasitology* **116**, 111–119.

Stensvold, C.R., Lebbad, M., Verweij, J.J., Jespersgaard, C., von Samson-Himmelstjerna, G., Nielsen, S.S. & Nielsen, H.V. (2010) Identification and delineation of members of the *Entamoeba* complex by pyrosequencing. *Molecular and Cellular Probes* **24**, 403–406.

Tantrawatpan, C., Intapan, P.M., Thanchomnang, T., Lulitanond, V., Boonmars, T., Wu, Z., Morakote, N. & Maleewong, W. (2012) Differential detection of *Trichinella papuae, T. spiralis* and *T. pseudospiralis* by real-time fluorescence resonance energy transfer PCR and melting curve analysis. *Veterinary Parasitology* **185**, 210–215.

Van De, N., Trung, N.V., Ha, N.H., Nga, V.T., Ha, N.M., Thuy, P.T., Duyet le, V. & Chai, J.Y. (2012) An outbreak of trichinosis with molecular identification of *Trichinella* sp. in Vietnam. *Korean Journal of Parasitology* **50**, 339–343.

Wu, Z., Nagano, I., Pozio, E. & Takahashi, Y. (1999) Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) for the identification of *Trichinella* isolates. *Parasitology* **118**, 211–218.

Zarlenga, D.S., Chute, M.B., Martin, A. & Kapel, C.M. (1999) A multiplex PCR for unequivocal differentiation of all encapsulated and non-encapsulated genotypes of *Trichinella*. *International Journal for Parasitology* **29**, 1859–1867.