Improved PCR-RFLP assay for the detection and differentiation between *Taenia solium* and *Taenia saginata* in faecal samples

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ABSTRACT

OBJECTIVE: To develop an improved PCR-RFLP method for differential diagnosis in faecal samples between *Taenia solium* and *Taenia saginata*. New primers were tested with better specificity in amplifying parasite DNA in faecal samples.

METHODS: Three different faeces extraction protocols (commercial QIAamp® DNA stool mini kit; guanidium thiocyanate/zirconium matrix method and DNA isolation based on a sequence-capture technique) were compared. The PCR-RFLP assay was used on faecal extracts from 71 known tapeworms carriers.

RESULTS: The three methods had similar sensitivities in fresh and stored (less than one year old) samples. The QIAamp® kit and the sequence-capture technique were the best extraction methods for faecal samples. However, extracts using the QIAgen kit gave rise to several aspecific bands in negative samples in contrast to the sequence-capture technique. The PCR-RFLP assay on faecal extracts from 71 known tapeworms carriers detected 20 samples positive for *T. solium* and 51 for *T. saginata*. Restriction with D*de*I enzyme gave clear and distinctive profiles for *T. solium*, *T. saginata* and *Hymenolepis nana*.

CONCLUSION: This is the first report on differentiation of all three parasites using a PCR-RFLP assay. The results of this study show that the newly developed assay will be very useful in epidemiological studies in endemic areas.

KEYWORDS**:** *Taenia solium*; *Taenia saginata*; *Hymenolepis nana*; PCR-RFLP; Diagnosis; Faecal samples; Ecuador.

INTRODUCTION

Taenia saginata and *Taenia solium* are medically and economically important tapeworms, causing bovine and porcine cysticercosis, respectively and taeniasis in man (WHO 2002, Yamasaki *et al*., 2002). Whereas cysticerci of *T. saginata* develop in cattle only, man can also be infected by the oncospheres of *T. solium* leading to neurocysticercosis, a major cause of epilepsy associated with considerable morbidity and mortality (Cruz *et al*., 1999; García *et al*., 2003). Traditional pig husbandry and poor hygienic conditions are key factors in the occurrence of human cysticercosis.

Early detection and identification of an adult *T. solium* infection is important in the control of neurocysticercosis. However, *T. solium* - cysticercosis remains often unnoticed, contrary to *T. saginata*, where the active migration of gravid proglottides is likely to alert the carrier.

Several methods to detect *Taenia* spp. in faecal samples have been reported i.a. visual detection of proglottids, detection of oncospheres by coproscopic examination and detection of copro-antigens by ELISA (Allan *et al*., 1990, 1996). However, these methods do not allow differentiation between *T. solium* and *T. saginata* unless followed by treatment and recovery of the somata, and differentiation by morphological and molecular techniques.

Rodríguez-Hidalgo *et al.* (2002) developed a technique based on PCR-RFLP of the 12s rRNA mitochondrial region for specific differentiation of taeniid somata.

The assay was further improved by designing a new reverse primer (ITMTr2), which in combination with T60F, was highly specific for *T. saginata* and *T. solium* somata in the absence of non-specific amplifications.

Extraction methods of *Taenia* DNA from faeces have been reported by Nunes *et al.* (2003), Yamasaki *et al.* (2004) and Nunes *et al.* (2005). The authors used two commercial extraction kits i.e. DNAzol reagent and QIAgen mini stool Kit and targeted different loci, using multiplex PCR or PCR-RFLP techniques. These assays lacked sensitivity and we report a new approach to analyse large number of faecal samples. Apart from studies on *Echinococcus granulosus* (Dinkel *et al*., 1998; Abbasi *et al*., 2003; Stefanic *et al*., 2004) there are no other reports on cestode DNA faecal extractions.

DNA extraction from faecal material is often giving rise to non-specific bands, false negatives or false positives because of the complex nature of the different sources of DNA and

PCR inhibitors like haemoglobin, bilirubin, bile salts, chelating agents and humic substances, present in faeces. (Widjojoatmodjo *et al.*, 1992; Shames *et al.*, 1995; Vansnick, 2004)

Several faecal DNA extraction methods have been developed (Walsh *et al.*, 1991; Boom *et al.*, 1990; Dinkel *et al.*, 1998; McOrist *et al.*, 2002; Nunes *et al.*, 2005), all using different methods for purification of bacterial, viral or protozoon DNA. McOrist *et al.* (2002) compared five different faecal extraction methods for the detection of bacterial DNA and concluded that the QIAamp® DNA stool mini kit (Qiagen, Germany) and the method described by Boom *et al.* (1990), gave the best results.

The objective of the present study is the development of a cheap, specific and highly sensitive test for the diagnosis and differentiation of *Taenia* DNA in faecal samples. Therefore a highly specific primer pair had to be developed, followed by the comparison of the different extraction protocols and improvements to the most appropriated method. The previous primers described by Rodríguez-Hidalgo *et al.* (2002) were highly specific for use on pure *Taenia* spp. somata but the same primers, when used on faecal extracts gave rise to non-specific bands, complicating the interpretation of the PCR results. The newly developed primers gave a single fragment of 371bp in the absence of non-specific bands.

MATERIALS AND METHODS

Faecal samples

A total of 76 faecal samples from different tapeworm carriers i.e. 71 with *Taenia* spp. and 5 with *Hymenolepis nana* from different regions in Ecuador (Highlands: Quito, Imbabura, Carchi, Tungurahua and Loja; Costal region: Manabí and Guayas) were collected. All samples were stored either as such or in an equal volume of ethanol 70% at -20°C. Carriers were identified by microscopy after concentration by Ritchie's formaldehyde-ether method (Ritchie, 1948); positive carriers were treated with a single dose of praziquantel (10mg/kg bw) to collect the adult parasites. These were diagnosed and compared using morphology and PCR-RFLP as described by Rodriguez-Hidalgo *et al.* (2002). Faeces, for PCR-RFLP analysis, were collected before treatment. In addition, 5 negative faecal samples from a non endemic area (Belgium) were used as controls and 14 negative faecal samples from Ecuador were used to determine the specificity of the new primer pair.

DNA-extracts from *Taenia* somata (*T. saginata* and *T. solium*), identified by morphology and GPI pattern, collected in Ecuador were used as positive controls.

Development of a new *Taenia* sp. primer pair on the 12s rRNA gene

Sequencing of the total 12s rRNA from a *T. saginata* somata sample from Ecuador was done by using TaenF primer (5'-GTTTGCCACCTCGATGTTGACT-'3) on the mitochondrial LsRNA partial segment and ITMTnR primer (5'-CTCAATAATAATCGAGGGTGACGG-'3) on the mitochondrial 12s rRNA partial segment, giving a 901 bp fragment.

The complete 12s rRNA sequence of *T. saginata*, was then aligned and compared with the *T. solium* and *Taenia saginata asiatica* complete mitochondrial genome described by Nakao *et al.* (2003) (Genbank: AB086256) and Jeon *et al.* (2005) (Genbank: NC004826) respectively, using the Clustal program (Fig 1).

Primers

A complete new semi nested primer set (nTAE, ITMF and ITMTnR) was designed by means of the following programs: Amplify (W.R. Engels, 1993). Right Primer, version M1.25 (R.Isaac, Biodesk, 1994). The specificity of primers was verified by blasting (Genbank http://www.ncbi.nlm.gov/) against all the DNA sequences present in the Genbank.

The primer pair, nTAE/ITMTnR was used in the first round and ITMF/ITMTnR or T60F/ITMTr2 in the semi-nested PCR. Sensitivity and specificity was compared with the primer pair T60F and ITMTr2 (Rodriguez-Hidalgo *et al.*, 2002). Primers nTAE and ITMTnR were biotinylated for use in the capture sequence method on faeces as described by Mangiapan *et al.*, (1996).

DNA extraction

Three different DNA extraction methods for faeces were used in a comparative test, i.e. commercial kit (QIamp® DNA stool minikit, Qiagen-Germany), the technique described by Mangiapan *et al.* (1996), modified by Vansnick (2004) and the extraction method developed by Boom *et al.* (1990) adapted by McOrist *et al.* (2002) and further modified in the present study.

Protocols

Qiagen kit

Approximately 180-220mg of faeces was suspended in 360-440µl of PBS plus 2% v/v Polyvinyl polypyrrolidone (PvPP) for 10 minutes at 100°C and then centrifuged at 12000*g* for 3 minutes. The supernatant was discarded and the pellet re-suspended in 1ml of Milli-Q water (Millipore, Belgium) then centrifuged at 12000*g* for 3 minutes. The pellet was finally lysed with a buffer provided in the kit (QIamp® DNA stool minikit, Qiagen, Germany) according to the manufacturer's instructions.

Technique of Mangiapan et al. (1996), modified by Vansnick (2004)

This technique is based on the specific capture of *Taenia* spp. DNA in faeces samples by the sequential addition of biotinylated oligonucleotides and avidin-coupled magnetic beads.

Briefly, 1 gram of faeces was homogenised in 2ml of PBS plus 2% v/v of PvPP in Falcon tubes. Samples were heated at 100°C for 10 minutes then centrifuged at 1200*g* for 30 minutes. The pellet was re-suspended in 2% v/v Tween20 solution in Milli-Q water (1ml per 100mg faeces) and approximately 8 glass beads (diameter 2mm) were added. Thereafter the samples were shaken on a vortex in a 1.5ml Eppendorf tube and the homogenised suspension left to settle for 30 minutes. The supernatant was then centrifuged again for 30 minutes at 1500*g*. The pellet was re-suspended in 1ml of Milli-Q water and transferred to a new 1.5ml Eppendorf and centrifuged for 5 minutes at 12000*g*. The modifications of the protocol during this study consisted in the use of zirconium beads (Merlin Diagnostics, cat#. 11079101z; diameter 0.1mm) instead of glass microspheres; the temperature during the capture of sequences being 50°C instead of 60°C and the use of a new tube during the wash step for a cleaner to transfer beads (to get rid of remnants of faeces adhering to the tube wall).

Modified extraction method (Boom et al. 1990)

100-300mg Faeces was dissolved in 200-600µl PBS plus 2% v/v PvPP and boiled for 10 minutes at 100°C. Samples were centrifuged at 12000*g* for 3 minutes, the pellet was re-suspended in 500µl lysis buffer (60mM Tris-HCl, pH7.4, 60mM EDTA, 10% v/v Tween, 1% v/v Triton X-100, and 1.6M Gu-HCl), and 40µl proteinase K. Samples were incubated overnight in a Thermomixer compact (Eppendorf, Koln, Germany) at 50°C and shaken at 1400rpm. Then, 500µl zirconium beads (Merlin Diagnostics, cat#. 11079105z; diameter 0.5mm) were added and incubated for one hour at ambient temperature. The pellet was washed three times in 5.5M guanidinium thyocyanate in 50mM Tris buffer (pH6.4); twice in 70% ethanol and once in acetone and thereafter dried at 60°C for 15 minutes. DNA was then eluted from the zirconium beads by incubating the beads in 100µl TE buffer at 48°C for 20 min.

Polymerase chain reaction

PCR was performed in a total volume of 25µl containing 5µl faecal extract as template and 20µl PCR mix containing 1µl Yellow sub™ (GENEO Bioproducts, Germany); 3.3µl milli-Q water; 12.5µl buffer (20mM Tris-HCl pH8.4; 100mM KCl; 0.2% v/v triton X-100; 1.5mM $MgCl_2$); 100pmol/µl of a mix of the 4 deoxynucleotide triphosphates (dNTP, final concentration: 0.2mM); 25pmol/µl of each primer and 0.4U *Taq*-polymerase Silverstar (Eurogentec, Seraing,

Belgium). For the semi-nested PCR, a final volume of 25µl i.e. 0.5µl from the first PCR round plus 24.5µl PCR mix, as described above except that (0.3U) *Taq*-polymerase was used.

The first PCR round and the semi-nested PCR were performed in a PTC-100 Tetrad Thermal cycler (MJ Research, U.S.) The first PCR round (nTAE/ITMTnR primers) consisted of an initial denaturation step at 94°C for 4 min, followed by 40 cycles consisting of 94°C for 45s, 56°C for 4 s and 72°C for 45s. The semi-nested PCR (with ITMF/ITMTnR primers) consisted of an initial denaturation step at 94°C for 4 min, followed by 25 cycles of 94°C for 45s, 56°C for 45s and 72°C for 45s.

Five microlitre of each amplified product together with a gene Ruler™ marker of 100bp (MBI Fermentas, GmbH, St. Leon-Rot, Germany) was separated by electrophoresis, using a Mupid-21 system (Eurogentec, Seraing, Belgium) in 2% w/v agarose and 0.04M Tris-acetate plus 0.002 M EDTA buffer for 20min at 100volts. The gel was stained with ethidium bromide (Sigma-Aldrich) for 30min and the DNA products visualised by ultraviolet light.

RFLP (Restriction Fragment Length Polymorphism), was carried out as described by Rodríguez-Hidalgo *et al.* (2002) using *Dde*I as restriction enzyme.

RESULTS

Alignment of the newly 901bp sequenced fragment of *T. saginata* with the *T. solium* (896bp) and *T. s. asiatica* (899bp) fragments, described by Nakao *et al.* (2003), and Jeon *et al.* (2005) was used to design new specific primers for use on faecal samples (Fig. 1).

The amplification results with these new primers showed a reduction of the non-specific bands in both positive and negative DNA extracts (data not shown) due to improved homology in the primers sequences. The primers amplified equally well DNA from Belgium and Ecuador isolated *T. saginata* and *Cysticercus cellulosae* used as a control.

The analysis of the new primers (Fig. 1) by the blasting program (Genbank) did not reveal any homology to sequences of human or parasite origin. However, there are different sources of DNA in faeces often leading to non-specific bands in both positive and negative extracts.

Fig. 1, also showing the primers developed in this study and used to amplify *Taenia* spp. DNA present in human faeces i.e.:

ITMTnR	5'-CTCAATAATAATCGAGGGTGACGG-3'
ITMF	5'-TGTGACAGGGATTAGATACCCCATT-3'
nTAE	5'-CGTGAGCCAGGTCGGTTCTTAT -3'

QIAgen, Mangiapan and Boom extracts from 76 faecal samples were analyzed with primer pairs ITMTnR/nTAE and ITMF/ITMTnR in a semi-nested PCR. Results of the amplification from the DNA extracts obtained by the 3 different methods are shown in Fig. 2. Amplicons of the expected size of 797 bp were obtained spanning a region from the 3' end of the LsRNA towards the end of the 12s rRNA for the first PCR and 372 bp from the 12s rRNA mitochondrial region in the semi-nested PCR. Five samples were found negative and correlated well with the results obtained on the faecal samples from patients, found negative on coprology. The 76 PCR-positive samples were also found positive by coprology i.e. 71 for *Taenia* spp. and 5 for *H. nana*.

Fourteen negative samples for *Taenia* spp. were used to determine the specificity of the new primer pairs. Both QIAgen and Boom protocols showed several aspecific bands while the Mangiapan extracts did not show any aspecific bands (results not shown).

The RFLP results (Fig. 3) showed a clear distinction among *T. solium, T. saginata* and *H. nana* profiles. The D*de*I enzyme profile for *T. solium* DNA consisted of bands of 325bp and 46bp; restriction of *T. saginata* DNA with D*de*I gave 4 bands: two of 165bp and 163bp and two lower bands of 25bp and 21bp, respectively. The small band differences cannot be differentiated with the resolution of a 10% acrylamide gel, appearing as a single band following silver staining. *H. nana* DNA does not give a restriction profile as it is not cut by the enzyme (Fig. 3).

Of the 71 *Taenia* carriers, 51 faecal samples were positive for *T. solium*, and 20 were positive for *T. saginata*.

Two samples (E36 and E61) were found positive on coprology for both *Taenia* spp. and *H. nana* eggs. The PCR-RFLP assay showed two different profiles with a band at 340 bp for *T. solium* and 373 bp for *H. nana* (Fig 3, lane 1 and 2). The *H. nana* profile was confirmed by digestion and DNA extraction of somata and subsequent PCR-RFLP analysis. Cloning and sequencing was obtained and 100% homology with a partial *H. nana* 12s rRNA fragment (Genbank: AB031361.1) was found by blasting the sequence against all Genbank sequences.

To analyse the effect of storage on the samples, twenty two faecal samples were randomly selected and stored at -20ºC, for variable periods with or without an preservative. The extraction was done using the 3 methods described in this study. The Boom extraction detected less positives as 8/22 (36.36%) samples became negative (E11, E19, E31, E36, E38, E40, E56 and E74) and only 3/22 (13.63%) faecal samples tested also negative using the Qiagen and Mangiapan extraction; (E11, E31 and E40).

DISCUSSION

The development of highly specific and sensitive primers was based on the results of a clustal alignment of the newly obtained sequence of the 12s rRNA *T. saginata* fragment with the complete mDNA of *T. solium* and *T. s. asiatica.* The primers amplified successfully all important cestode DNA for which man is a natural host e.g. *T. solium, T. saginata, T. s. asiatica* and *H. nana*. According to the *Hymenolepis diminuta* sequences of the Genbank, our primers will not amplify this sequence which contrary to *H. nana* is a very rare parasite of man. Differential diagnosis of all three *Taenia* somata by PCR using different restriction enzymes has been possible (Pers. comm. Geysen, 2005)

Following treatment, only adult *Taenia* somata were recovered, differentiated and characterized on morphological grounds and confirmed using the PCR-RFLP assay as described by Rodríguez-Hidalgo *et al.* (2002). Analysis of the faecal extracts correlated perfectly with somatic extracts and the morphological observations (data not shown). Out of 71 positive *Taenia* carriers, two samples (E36 and E61) containing mixed infestations with *T. solium* and *H. nana* oncospheres showed two bands after PCR-RFLP analysis (Fig 3), one for *T. solium* (around 325bp) and one for *H. nana* (around 370bp). This study shows that the presence of *H. nana* infections, a common parasite in Ecuador, is not a confounding factor in the detection of *T. solium* or *T. saginata* by this PCR-RFLP assay since the 370bp PCR fragment of *H. nana* does not get restricted by the D*de*I enzyme. In spite of *H. nana* being a common but apathogenic parasite in humans, it has not been consider in previous studies notwithstanding that it could interfere with the results.

In areas where both *T. solium* and *T. saginata* are present, it is of utmost importance to identify the parasite to the species level (Abbasi *et al.*, 2003). The most commonly used diagnosis for taeniasis is microscopic detection of oncospheres, but sensitivity is low i.e. 38% (Flisser *et al.*, 1999). Allan *et al.* (1990, 1996) developed a copro-antigen assay to detect *Taenia* spp excretion and secretion products in faeces with a sensitivity reported around 98%. None of these methods however, allows differentiation between *T. solium* and *T. saginata*.

Recently, several methods based on molecular or immunodiagnostic techniques were developed to study the epidemiology of taeniasis and cysticercosis.

Wilkins *et al.* (1999) used specific antigens to detect antibodies against adult *T. solium* in serum samples by Western blot. This test has a sensitivity of 95% and specificity of 100%. No cross-reactions were found in samples from *T. saginata* and other cestode infections. However,

one sample, from a patient with Neurocysticercosis (NCC) without intestinal *T. solium*, tested positive. The major disadvantage of this test is the detection of residual antibodies from past exposure which might give false positives in patients with NCC (Ito and Craig, 2003).

Molecular based detection of *Taenia* implies the isolation and purification of all parasites DNA present in faeces. This has been proven difficult due to high concentrations of inhibitors present in faeces. McOrist *et al.* (2002) compared 5 different DNA extraction and purification methods for bacterial DNA including the Qiagen mini stool kit (QIamp® DNA stool minikit, Qiagen-Germany) and the extraction as reported by Boom *et al.* (1990). Their reports showed that the Qiagen and Boom extraction were the most sensitive tests to detect bacterial DNA in faeces. Therefore, these two methods together with the Mangiapan technique were tested in this study.

In our test, non-specific bands were found in the negative faecal samples extracted with the QIAgen or the Boom technique and are most likely due to the complex DNA mixture found in faeces. On the other hand, the Mangiapan extraction did not show any aspecific bands probably due to the selective capture of only *Taenia* DNA from faeces using the biotinylated nTAE/ITMTnR primers.

Samples more than one year old yielded fewer positives in the Boom extraction, whereas QIAgen and Mangiapan showed the same level of sensitivity, even with samples more than 30 months old. Probably, the Boom method is better in extracting degenerated DNA due to high concentrations of inhibitors, proteolytic enzymes or products of conservation or the extraction products might be less affected by the extraction method.

This study reports on the improvement of the extraction efficiency for *Taenia* spp. DNA in faeces based on the Mangiapan (modified by Vansnick, 2004) technique. This is a valuable alternative to the QIAgen method with the added advantage of being far cheaper. Being also user-friendly and highly sensitive, this method is on the other hand more time consuming because of the length of the incubation periods i.e. extraction of a sample requires ±24 hours whereas QIAgen takes around ±50 min.

We can conclude that the new PCR-RFLP assay in combination with an optimised and low cost DNA extraction and purification method is a test with great potential in analysis of faecal samples for specific diagnosis of *Taenia* spp.

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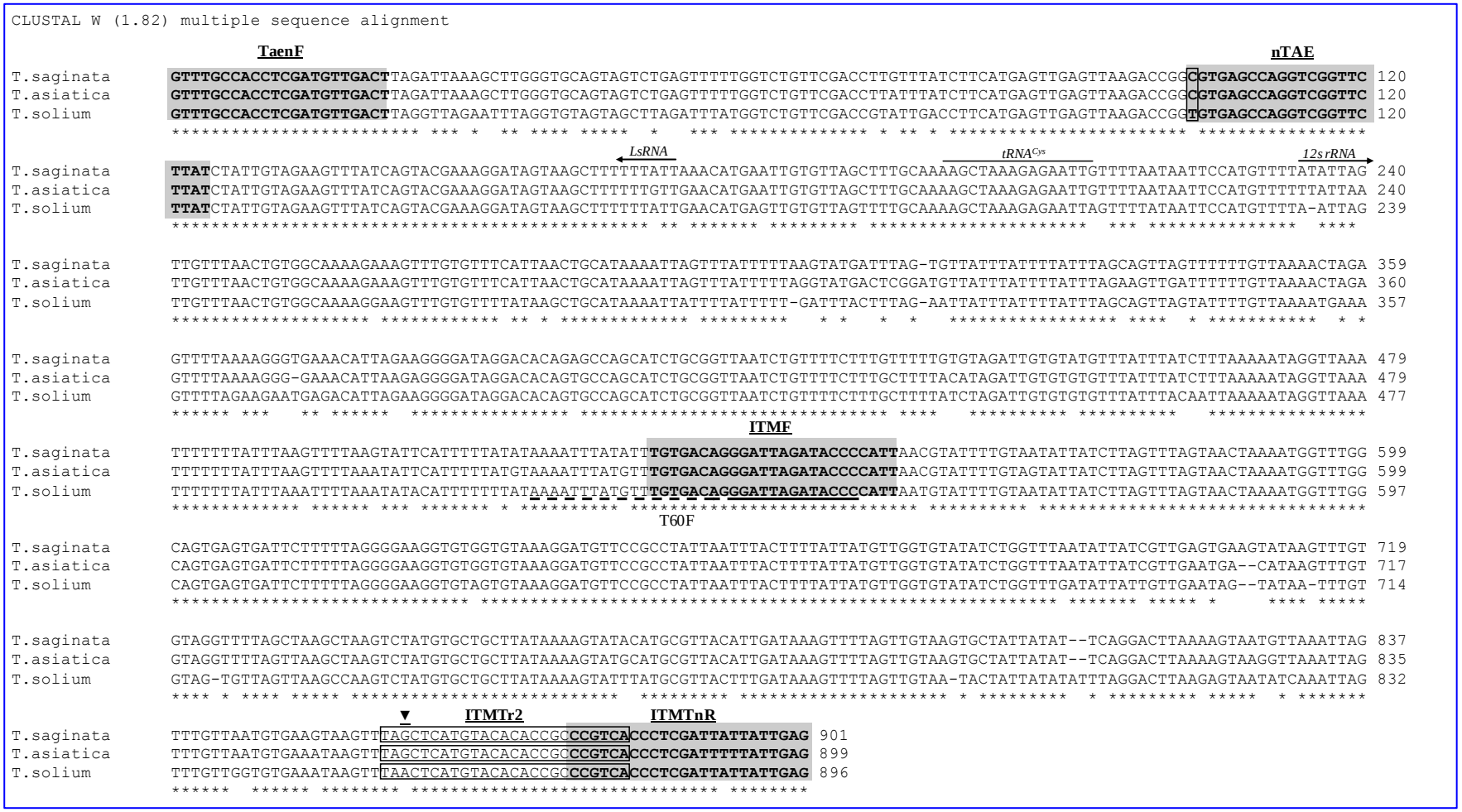

Figure 1. Multiple sequence alignment of *T. solium*, *T. s. asiatica* and *T. saginata* mDNA fragments. *T. saginata* specific-901bp was amplified using TaenF/ITMTnR primers. The primers used in this study are marked in bold and the end of the LsRNA and start of the 12s rRNA fragments are indicating by arrows respectively. nTAE and ITMF primers are developed during this study. The 3th base (▼) of ITMTr2 described by Rodriguez-Hidalgo et al., 2002 shows a difference. T60F (TTAAGATATATGTGGTACA**GGATTAGATACCC**) and ITMF primers are conserved in the last 13bp, shown by the bp in bold. Stars indicate consensus sequences, gaps indicate differences, - are delections.

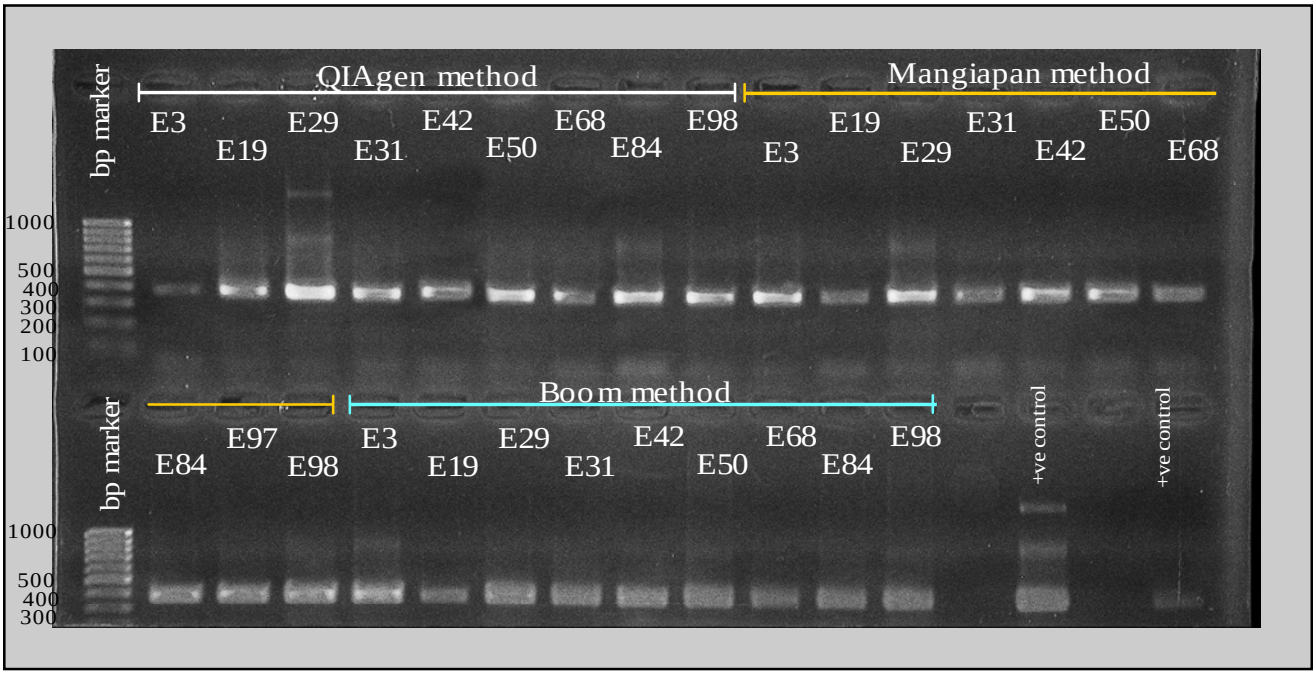

Figure 2. PCR results corresponding to the different DNA extraction methods i.e. QIAgen, Mangiapan and Boom assay. Amplification of the mitochondrial 12s rRNA segment from taeniids in faeces, using primers ITMTnR/nTAE (first round) and ITMTnR/ITMF (semi-nested). Analysis is performed in a 2% agarose gel and stained with ethidium bromide. The marker is a 100-bp DNA ladder. Positive samples show bands at ± 370bp.

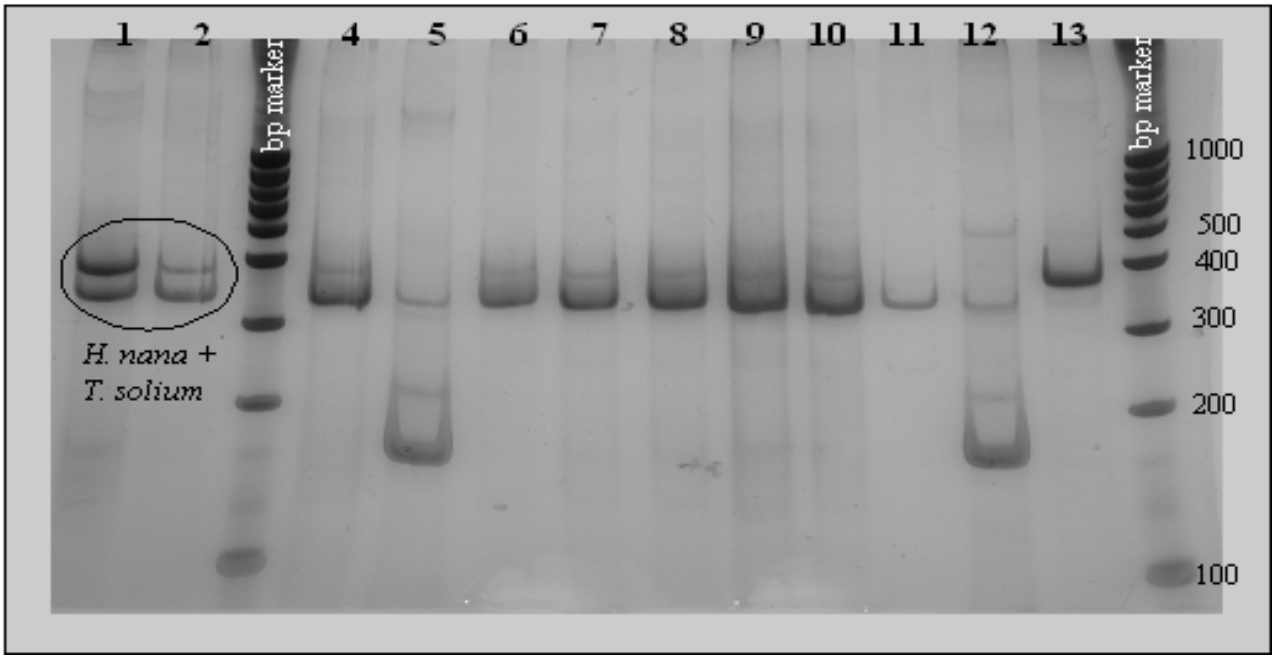

Figure 3. D*de*I enzymatic restriction of the 12s rRNA segment. Restriction results positive *T. solium* (lane 4, 6-11); *T. saginata* (lane 5 and 12); *H. nana* (lane 13) and the carriers whit both *T. solium* and *H. nana* (Lane 1 and 2) as described in table 1. High single bands (±325bp) correspond with *T. solium* profiles, whereas the low two bands (±160bp) are *T. saginata* profiles. *H. nana* is not restricted (±370bp). Nonspecific bands are absent. Analysis is performed using 10% polyacrylamide gel and stained with a Silver staining kit. The marker is a 100-bp DNA ladder

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