ABSTRACT

Considerable economic loss occurs due to helminth parasites in country fowl throughout the world. Though less emphasis has been placed on utilising DNA elements to study cestodes of veterinary importance, correct diagnosis is, nevertheless, critical to proper treatment and control. These are able to induce structural, biochemical and immunological changes in the host like inappetence, diarrhoea, dull hair coat and ostertagiosis. At present, control is almost exclusively based on anthelmintics. Hence, Genomic DNA analysis plays a vital role in characterizing and classifying cestode parasites. The present study revealed restriction digestion analysis of genomic DNA of *Raillietina echinobothrida* reference isolates collected from country chicken “*Gallus domesticus*” from Zambazaar market, Triplicane, Chennai (Tamil Nadu) using 9 restriction endonucleases enzymes namely HindIII, EcoRI, BamHI, BglII, HaeIII, XhoI, SfiI, NheI and Hinfl revealed some differences in the restriction profile of DNA in the chick isolates. Lambda HindIII marker was used to compare the genomic DNA bands of *R. echinobothrida* formed in the gel. *R. echinobothrida* digested with the enzymes like EcoRI, BamHI, BglII, HaeIII and XhoI compared with 23kb of lambda HindIII marker and most of them showed streaky and smeared formation of DNA and SfiI, NheI and Hinfl showed no bands.

**KEYWORDS:** *Raillietina echinobothrida*, genomic DNA analysis, restriction enzymes.
INTRODUCTION

*Raillietina echinobothrida* belonging to the family of *Davaineidae* are of common occurrence. According to Southwell (1930) *Raillietina* species are considered as serious pathogens. The adult tapeworms are quite variable with reference to the effects they produce on the hosts. Loss of production, slow development and increased susceptibility to other disease can however be expected in farms with a heavy tapeworm infection. Laying birds show decreased or even complete termination of egg laying. Older birds appear to acquire resistance even though they may harbour considerable numbers of parasites. Leg weakness and paralysis have frequently been attributed to tapeworm infection. Capillary congestion, cellular infiltration and connective tissue proliferation in intestinal wall and fibrosis are other conditions which have been associated with tapeworm infection (Nath and Pande, 1963).

The tapeworm *Raillietina* species is one of the most pathogenic parasites which occur in the small intestine of chicken and turkeys in most parts of the world (Ramesh Kumar *et al.*, 2007). Nodular disease caused by the cestode, *Raillietina* species is widely recognized as an increasingly important disease in chicken in developing countries. Nodules and hyperplastic enteritis may develop at the site of attachment in the intestine of chicken. This phenomenon is named “nodular tapeworm disease” and may occur in heavy infections.

Infections with *R. echinobothrida* are found in chicken and turkeys. It is cosmopolitan in distribution (Anders Permin and Hansen (1999). Studies and reports have revealed that among the diverse species of *Davaineidae* cestode parasites that inhabit the intestine of fowl *R. echinobothrida* is the most harmful one, as appraised by the pathological implications. They absorb glucose and galactose and stored them as glycogen as well as absorbed amino acids, polypeptides and proteins (Thomas C. Cheng, 1973).

Molecular approaches are the most effective and accurate means for the detection of many organisms and for screening of genetic variation among populations (Wongsawad and Wongsawad, 2010). Economic losses due to helminth parasites in sheep throughout the world are considerable (Irfan-ur-Rauf Tak *et al.*, 2013). The features of the host – parasite relationships among the taeniid cestodes have formed the basis for vaccine development. Research on the development of vaccines to prevent infection with cestode parasites has brought focus on the taeniid cestodes (Lightowlers *et al.*, 2002). Cestodes of the family *Davaineidae* in their adult form parasitize a variety of hosts including birds and mammals (Wardle and McLeod, 1952 and Yamaguti, 1959). Cestode parasites are worldwide chronic
zoonosis affecting humans as well as domestic and wild mammals, which has been reported as a prioritized neglected disease by the World Health Organisation. No genomic data, comparative genomic analyses or efficient therapeutic and diagnostic tools are available for this severe disease. The information presented in this study will help to understand the peculiar biological characters and to design species-specific control tools (Maldonado et al., 2017).

Understanding the life cycle and pathogenesis of zoonotic helminths is extremely important because a majority of the infections are acquired from the livestock animals. These animals (including birds) live in close association with the human beings and serve as a reservoir of infections (Biswal et al., 2014). Not surprisingly enough in most of these infections the causative agents go unrecorded due to failure of identification of the parasite. Hookworms are known to be host-specific (Mabvuure et al., 2015).

The restriction enzyme, Phosphoenolpyruvate carboxykinase (PEPCK) involved in gluconeogenesis in higher vertebrates opposedly plays a significant role in glucose oxidation of the cestode parasite, *Raillietina echinobothrida*. Considering the importance of the enzyme in the parasite and lack of its structural details, there exists an urgent need for understanding the molecular details and development of possible modulators. Hence, in this study, PEPCK gene was obtained using rapid amplification of cDNA ends and various biocomputational analyses were performed. Homology model of the enzyme was generated and docking simulations were executed with its substrate, co-factor and modulators. Computer hits were generated after structure- and ligand-based screening using Discovery Studio 4.1 software; the predicted interactions were compared with those of the existing structural information of PEPCK. In order to evaluate the docking simulation results of the modulators, PEPCK gene was cloned and the overexpressed protein was purified for kinetic studies. Enzyme kinetics and in vitro studies revealed that out of the modulators tested, tetrahydropalmatine (THP) inhibited the enzyme with lowest inhibition constant value of 93 nm. Taking the results together, Asim Kumar Dutta et al., (2016) concluded that THP could be a potential inhibitor for PEPCK in the parasite. The present investigation was undertaken to analyse the nature of sequence variation and the effect of 9 restriction enzymes (enzymes namely HindIII, EcoRI, BamHI, BglII, HaeIII, XhoI, SfiI, NheI and HinfI) of the genomic DNA of *R. echinobothrida*. 
MATERIALS AND METHODS

1. Animal procurement

The cestode parasite, *Raillietina echinobothrida* (Megnin, 1881) were obtained alive from the intestine of naturally infected and recently killed country fowl “*Gallus domesticus*” collected from Zambazaar market, Triplicane, Chennai (Tamil Nadu). Then the collected specimens were rinsed in distilled water to render them free from intestinal contents and kept in separate petridishes for identification. The entire worms were spread out on a glass pane and the length of the worms was measured. The immature, mature and gravid proglottid regions of both the worms were identified and separated and were homogenized with 2ml of distilled water and used for biochemical analysis.

9 Restriction enzymes - HindIII, EcoRI, BamHI, BglII, HaeIII, XhoI, SfiI, NheI and HinfI were used for restriction digestion analysis of genomic DNA of the cestode parasite, *R. echinobothrida* infecting the intestine of the country chicken (*Gallus domesticus*).

Ahmed and Abdel-Moaty (2011) reported that *Cotugnia polycantha* infecting doves, *Streptopelia senegalensis*, differs from that infecting pigeons, *Columbia livia domestica*. Sperm ultrastructure was used as a new tool for identification and *C. Polycantha* infecting two different hosts, doves *S. senegalensis* and pigeons *C. livia domestica*, were compared using random amplified polymorphic DNA (RAPD) analysis for differentiation between them. In the present study the genomic DNA of two cestode parasites were studied using multisource genomic DNA, mini-prep kit and electrophoretic study by Agarose gel electrophoretic method (Sabry E. Ahmed, 2012).

2. Genomic DNA isolation

Genomic DNAs were isolated on a small scale from 1ml of adult worms using multisource genomic DNA, Mini-Prep Kit, Axgene Biotechnology, U.S.A Cat. No. 110420 - 25, according to manufacture manual. Gene extraction studies do not reveal protein turnover patterns and protein modifications that may be involved in signalling and communication, protein transport and targeting important phenomenon (Savithry Natarajan et al., 2014). The isolation of total genomic DNA from *R. echinobothrida* was carried out under two steps:

a. Extraction of genomic DNA

10.1 grams of the sample of the parasite was weighed and mixed well with 10ml of lysis (NaCl, Tris HCl (pH 8.0), EDTA, SDS) buffer till the tissue was blended with the buffer. The tissue was left overnight at room temperature. 5ml of saturated phenol and 5ml of saturated
chloroform were added and mixed well. The tube was gently inverted to mix the aqueous and organic phases until a cloudy emulsion was formed. The tube was then centrifuged at 12000 rpm for 15 minutes to separate the phases. After centrifugation the upper aqueous phase containing the DNA and the lower organic phase, containing the denatured protein and other contaminants were observed. The aqueous phase with DNA was transferred to a 1.5ml tube and the organic phase was discarded.

b. Precipitation of genomic DNA
1.1ml of 3m sodium acetate (pH 4.8) was added to 11ml of DNA solution. The tube was capped and inverted to mix well and the DNA was precipitated with the help of 25ml of absolute ice cold ethanol. The tube was centrifuged at 12,000 rpm for 15 minutes and the supernatant was discarded. The pellet of DNA was washed with 70% ethanol and centrifuged at 12,000 rpm once again for 15 minutes. The supernatant was discarded and the precipitated DNA was removed until the excess ethanol has drained away and evaporated. Then TE buffer was added and the agarose gel electrophoresis was carried.

3. Restriction endonuclease enzyme digestion
Genomic DNA of *R. echinobothrida* was digested with 9 restriction endonuclease enzymes using reaction conditions of the enzymes. The restriction enzymes tested were HindIII, EcoRI, BamHI, BglII, HaeIII, XhoI, SfiI, NheI and Hinfl. A typical enzyme reaction consisted of 2 micrograms of genomic DNA, 5 microlitres of restriction buffer, 0.4 microlitres of each restriction enzyme and 4.6 microlitres of sterile water. The enzymatic reaction was incubated using a water bath at 370C for 35-45 minutes.

4. Agarose gel electrophoresis
Agarose gel Electrophoresis was carried out according to the procedure as outlined by Sambrook and Russel (2001). The DNA analysis of the cestode samples was started by preparing a 0.7% agarose gel to run the samples. TAE was used as the buffer with a concentration of 40mM tris (pH7.6), 20mM acetic acid and 1mM EDTA was used as the marker gene. Bromphenol blue was used as the marker dye at a concentration of 1μl for every 5μl of DNA sample. Ethidium bromide was used as the visualization dye which was mixed in the buffer tank at 0.5mg/ml. The solution was well stirred to disperse ethidium bromide. 20μl of DNA sample were loaded into lane 1 and the marker gene λ Hind III sample was loaded into lane 2. The gel electrophoresis was carried out separately for the two cestode
parasites. Current was supplied typically at 100V for 30min. After electrophoresis the gels were observed under a UV lamp wearing a protective gear.

5. UV – Spectrophotometer for sample purity
The double beam UV-Spectrophotometer (Shimadzu Corporation) connected to PC UV1650 was used for sample testing and all further operations were done using computer keys. Thus by using UV spectrophotometer the sample DNAs of the cestode, *R. echinobothrida* were assessed for their purity and the ratios of pure preparations of DNA of both the samples were found to be close to 2.0.

RESULTS
The picture of agarose gel electrophoresis of genomic DNA of *Raillietina echinobothrida* was revealed in the lane 2 of Plate I.

![PLATE – 1](image)

In the present study, the DNA of *R. echinobothrida* was observed as a bright intense orange coloured band. The band can be well differentiated by its relative mobility. The results of agarose gel electrophoresis revealed that the DNA of *R. echinobothrida* was found to be a slow moving fraction and had a single fraction. This was found to be homologous to the HindIII 23kb DNA marker lane 5 of Plate II. Because of its high molecular weight it migrated slowly. With the knowledge of observing the colour developed and the thickness of the band, the DNA content of *R. echinobothrida* was found to be heavier.

The picture of agarose gel electrophoresis of *R. echinobothrida* with genomic DNA and restriction endonuclease digest of genomic DNA was revealed in the Plate II.
The lane 1 of Plate II showed the migration of genomic DNA fragment in 0.8% agarose gel of the cestode species, *R. echinobothrida* at 23kb. The DNA appeared as a single thick intense orange coloured band moving close to 23kb of the lambda HindIII marker found in lane 5.

The lane 2 of Plate II did not reveal the fragments of genomic DNA sample of the cestode species, *R. echinobothrida* digested with the restriction endonuclease enzyme HindIII.

The lane 3 (Plate II) showed the fragments of genomic DNA sample of the cestode species, *R. echinobothrida* digested with the restriction endonuclease enzyme EcoRI. The genomic DNA restriction sites appeared as streaky and smearing coloured band starting at 6kb and moving down to the end of the lane.

The lane 4 (Plate II) showed the restricted band of genomic DNA sample of the cestode species, *R. echinobothrida* digested with the restriction endonuclease enzyme BamHI. The genomic DNA restriction sites appeared as streaky and smearing coloured band from 4.3kb approximately.
The lane 5 (Plate II) showed lambda HindIII marker as a control, which is a mixture of molecules of known sizes. The lambda HindIII marker DNA is a mixture of 23kb, 9.5kb, 6.5kb 4.3kb, 2.3kb and 2.0kb. Fragments of linear DNA migrated through agarose gel and separation of the components from the marker mixture formed distinct bands in the gel one after the other. The distance of the band travelled is approximately inversely proportional to the logarithm of the size of the molecule.

The lane 6 (Plate II) showed the fragments of genomic DNA sample of the cestode species, *R. echinobothrida* digested with the restriction endonuclease enzyme BglII. The genomic DNA sample of *R.echinobothrida* digested by BglII was very faint and streaky from 4.3kb to 4kb of lambda HindIII marker in lane 5 (Plate II).

The lane 7 (Plate II) showed the fragments of genomic DNA sample of the cestode species, *R. echinobothrida* digested with the restriction endonuclease enzyme HaeIII. The genomic DNA sample of *R.echinobothrida* digested by HaeIII was seen to be very faint and streaky smear below 1kb of lambda HindIII marker.

The lane 8 (Plate II) showed the fragments of genomic DNA sample of the cestode species, *R. echinobothrida* digested with the restriction endonuclease enzyme XhoI. The genomic DNA sample of *R. echinobothrida* digested by XhoI was observed to be very faint and streaky smear from 6kb onwards.
The *lane 1* in **Plate III** showed the migration of genomic DNA without the restriction enzymes. In addition to this, the genomic DNA sample of cestode species, *R. echinobothrida* digested with the restriction endonuclease enzymes like SfiI, NheI and HinfI (lane 2, lane 3 and lane 4) in **Plate III** showed no restriction sites.

**DISCUSSION AND CONCLUSION**

Restriction digestion analysis of DNA with restriction endonuclease enzymes is a very useful and reliable technique to discriminate highly similar parasitic strains within the same species or subspecies. With the discrimination of the strains of parasites using this technique, a way could be laid to get the knowledge of the genetic nature of the parasites which in turn leads to the way of drug design and vaccine development against the parasite. The direct study of nucleotide variation by DNA sequencing or restriction endonuclease analysis can provide an extremely sensitive measure of genetic variation, as long as the DNA sequence analysed is appropriate to the level of variation. But in the case of *R. tetragona* DNA from the referral chick isolate could be restricted by Bgl I, Pvu II and Dra I and no restriction site could be fixed with the restriction enzyme Hinf I and restriction digestion of genomic DNA of *R. tetragona* with the restriction enzymes Bgl I and Pvu II formed a band above 23 Kbs of Lambda Hind III marker and streak formation below 2Kbs. Whereas, restriction digestion with Dra I formed a single band which was above 23 Kbs of Lambda Hind III marker. Restriction digestion of genomic DNA of *R. tetragona* with the restriction endonuclease enzyme Bgl I showed a small light band above 23kbs of Lambda Hind III marker and streak formation below 2kbs (Manju Bashini et al., 2015). But in the case of cattle isolate *Echinococcus granulosus*, DNA digested with Bgl I fixed no restriction site as reported by Reddy and Rao, (1998) and restriction digestion of genomic DNA of *R. tetragona* with the restriction endonuclease enzyme Hinf I also showed no restriction sites. Whereas Reddy and Rao, (1998) reported 3 restriction sites in *E. granulosus* of buffalo isolates as 3.4, 2.2 and 2.1 kb DNA bands within the smear of DNA with Hinf I and cattle DNA showed uniform smearing with low resolution. In the present investigation, studies on cestode DNA macromolecule of *R. echinobothrida* provide the basic understanding in the isolation of genomic DNA and restriction digestion of DNA. Restriction fragments make it possible to locate the positions of restriction sites relative to one another on a DNA molecule. Detecting genetic variation and significance of genetic variation within strains is the relative efficiency of electrophoresis and restriction endonuclease analysis of DNA. Mc Manus and Rishi (1989) used three six- base enzymes in their study for *Echinococcus granulosus* of British sheep
origin. They found an average number of resolvable restriction sites of seven with their rDNA probe and nine with their randomly cloned probe Cuesta – Bandera et al., (1988) reported distinct hybridization pattern of restriction endonuclease derived DNA fragments of *E. granulosus* from Spain between sheep and donkey strains. The experimental studies of restriction digestion of genomic DNA of *R. echinobothrida* has been made to provide the fundamental steps in the gene cloning process and restriction mapping of the cestode parasite. Restriction mapping is a very useful and necessary technique for any type of cloning project. Further knowledge of genomic DNA isolation and restriction endonuclease studies help in the identification of species, detection of species variation at the molecular level and in phylogenetic classification of cestode parasites. Assessment of the biological characteristics and other intrinsic criteria of *R. echinobothrida* are warranted to corroborate the present DNA findings. Further studies including DNA probes, restriction enzymes and restriction mapping of genes will be required to substantiate these observations.

Besides the cost effectiveness, the acceptability of methods to the medicos is of prime importance if these at all can be used for the benefit of the humans. Ante mortem diagnosis and instruments for recording helminth infections in field based studies are equally important for proper data recording. Diagnosis of pork/beef/meat for presence of any helminth and/or their developing stages before being made available to man (for consumption) is vital from the view of food-safety issues. Technologies in this field are also wanting. Literature shows that studies so far has been unidirectional aimed at developing sensitive and specific methods that has found place only in a researcher’s laboratory or academic papers. Applicability and feasibility has been only rarely considered. These genetic engineering studies will really be worth when it becomes accessible to common man. Research should thus focus on how to make these technologies available to the common mass rather than being restricted to only academic studies.

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