

Taenia Hydatigena Cysticercus and Its Toxic Effects on Liver and DNA Profile in Sheep

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Abstract: Cysticercosis of farm and wild animals is caused by the larval stages (metacestodes) of cestodes (tapeworms). Damage to tissues has been described in the cells of host infected with different species of *Taenia*. This damage is due to the increased amount of reactive oxygen radicals, which cause oxidative stress. Lipids, hepatocytic proteins and DNA are among the structures that are mostly affected by reactive oxygen and nitrogen species; resulting in functional and structural abnormalities, particularly in the liver. The objective of this work was to evaluate the impact of *T. Hydatigena* on oxidative modifications of hepatocytes in sheep. Liver samples were taken from domestic and imported sheep slaughtered in local abattoirs in Saudi Arabia. The effect of oxidative stress on hepatic tissue was studied by measuring biochemical parameters such as: glutathione, malondialdehyde, catalase activity, and glutathione S-Transferase assays. In addition, RAPD-PCR was carried out using three primers (AP₁, AP₂ and AP₃) to investigate the changes in DNA fingerprints due to parasitic infection. Percentage of genetic variations were measured in the different sheep samples, which showed that the DNA profile of infected sheep were different from health sheep between 51-62%. Generally local sheep were more affected than imported ones.

Key Words: liver toxicity, cysticercosis, biochemical measurements, *Tania hydatigina*, RAPD-PCR.

I. Introduction

Sheep comprise one of the most important livestock for Saudi Arabia and play an important role in the livelihood of a large portion of small and marginal farmers and nomads (Alshaikh *et al.*, 2004). Parasites pose serious problems to the livestock industry, and as such are well worthy of the detailed attention required to explain how they cause disease (Dargie, 1980). Sheep liver is the target organ for many parasitic infections. It can be parasitised with different helminth infections such as *Fasciola spp.*, *Dicrocoelium* and larval cestode infections such as *hydatid cysts* and *cysticercosis* (Thompson and Lymbery, 1995; Radfar *et al.*, 2005; Ahmadi and Badi, 2011).

Infections with the larval stage of some species of *Taenia* are not only of public health importance, but also of veterinary significance because they cause economic losses due to banning infected offal or meat. The infection may lead to less production, or even death of the animals in cases of heavy infections.

Some stressors such as Heat, cold, drought, dehydration, infection, etc., result in cellular oxidative stress which is an imbalance between oxidation and anti-oxidation (reduction). Oxidative stress is a common mechanism contributing to hepatic damage and fibrogenesis in a variety of liver disorders. It can be monitored with several biomarkers (anti-oxidants and pro-oxidants), which can be assessed in plasma, erythrocytes, and liver. Superoxide dismutase, catalase, peroxidase, monoamine oxidase, glutathione reductase activities, glutathione, etc., are considered as good serum markers of oxidative stress (Passi *et al.*, 2001; Kataria and Kataria, 2005). Earlier studies have been carried out regarding the toxic effects and the oxidative stress of liver caused by parasitic infection in different hosts. *Echinococcus granulosus* and *E. multilocularis* (Smyth, 1964), *Fasciola hepatica* (Castro, 1996; Calleja *et al.*, 2000), *Plasmodium falciparum* (Mikolajczak and Kappe, 2006). Recently, several studies were carried out on the impact of parasitic infection on the hepatotoxicity and liver apoptosis. Mou *et al.* (2012) investigated the effects of *Taenia asiatica* and *T. saginata* on hepatocyte apoptosis in experimentally infected pigs. Also, Zebeda *et al.* (2016) studied the apoptosis in spleen CD4T cells, TGF- β and Fox p3 gene expression in mice induced by *T. crassiceps* factor. The present study aimed to evaluate the oxidative modifications of sheep hepatocytes infected with metacestodes of *Taenia hydatigena* in local and imported sheep using a variety of relevant biochemical techniques. Also DNA profile of infected sheep was followed in comparison with normal ones using RAPD-PCR assay.

II. Materials And Methods

Chemicals

All common chemicals used were purchased from one of the following suppliers Sigma Co. (St. Louis, MO, USA). All other reagents were of the highest grade commercially available. (All chemicals used in the study were of analytical grade).

Samples collection and examination:

Total of 120 Liver samples were collected from domestic and imported sheep slaughtered in local abattoirs in Medina (Medina slaughterhouse No. 7) in the Kingdom of Saudi Arabia during the periods of October 2013 to May 2014. The countries of origin of most of the imported samples are Sudan and Somalia.

The samples included 20 healthy liver used as control and 100 naturally infected liver with larval cestodes. Out of the 100 naturally infected samples, 23 samples were collected from local sheep while, 71 infected liver were collected from Sudanese sheep and 6 Somali samples. The identified samples infected with cysticercus of *T. hydatigena* were chosen from local and imported sheep. (10 samples for each).

After microscopic examination, the number, shape and arrangement of rostellar hooks were determined. Total lengths of large and small hooks were also measured using a calibrated eyepiece micrometer under oil immersion objective (Sweatman and Williams, 1963). The number of hooks was determined and the length of the hook and hook parts were measured as described by Hobbs *et al.* (1990).

The collected liver samples were kept in two vials, one of them was stored at a temperature of -86 °C until used for the physiological tests. The other part was kept in 70% ethyl alcohol for subsequent DNA analysis.

Preparation of liver homogenate for biochemical investigation

The hepatic tissues were homogenized in 10ml ice-cold phosphate buffer (100 mM, pH 7.4). The homogenized tissue (1g of tissue /10ml PBS) was centrifuged at 4000rpm for 15 minute. The supernatant was separated and stored at -80°C for determination of oxidative stress markers and cellular defenses (MDA, GSH, GST and CAT).

Assessment of oxidant/antioxidant activities

Catalase (CAT), glutathione (GSH), glutathione S-transferase and malondialdehyde (MDA) levels were tested in the hepatic tissue homogenate.

Bradford Protein Assay

The solutions required for the Bradford protein assay was prepared using the following ingredients: phosphate buffer (pH 7.4), reactive Bradford agent, bovine serum albumin. The standard protocol for preparation was to put 5 µl of phosphate buffer or different concentrations of BSA in the wells and adding 250 µl of the Bradford reagent to it. The samples were then prepared by diluting the supernatant five times, using 5µl of the sample, and 20µl of the phosphate buffer. After this, 10 µl of the diluted extract and 250 µl Bradford reagent were added to ELISA plates. The spectroscopic absorbance was read after 2 minutes at the wavelength of 595 nm.

Glutathione (GSH)

The GSH assay was prepared according to the method described by Moron *et al.* (1979). The assay is based on the reaction of GSH with DTNB (also known as Ellman's reagent) that produces the TNB chromophore, which has a maximal absorbance at 412 nm, and the oxidized glutathione TNB adduct (GS–TNB). The rate of formation of TNB, measured at 412 nm, is proportional to the concentration of GSH in the sample. Glutathione is used as a standard to calculate the amount of GSH expressed as mmole of GSH/mg proteins. The solutions required for this assay were: phosphate buffer (pH 8), 5% TCA solution, used for protein precipitation, 10 mM DTNB solution (dissolved in phosphate buffer). 0.01M glutathione (GSH) solution was used as standard. The procedure of preparation was as follows: 50 µl of solution (TCA) was added to 50 µl of extract, 50 µl TCA 5% was combined with 50 µl extract. The sample was centrifuged for 10 minutes at 14000 rpm. The supernatant was then analysed for GSH content, which was done by adding 80µl extract, 120 µl phosphate buffer and 20 µl DTNB to the ELISA plate and measuring the absorbance at a wavelength of 412 nm.

Malondialdehyde Assay

The MDA assay is based on the reaction of a chromogenic reagent, 2-thio-barbituric acid, with MDA at 25°C. One molecule of MDA reacts with 2 molecules of 2-thio-barbituric acid via a condensation to yield a chromophore (pink colour) with absorbance maximum at 532 nm. In this study, the MDA levels were determined according to the method of Ohkawa *et al.* (1979). Levels of MDA were calibrated against a MDA standard curve, and expressed as mmole/mg protein. The solutions used were: 85% phosphoric acid, TBA 30 mM (MW=144.15g/mole), KCl 154 mM (MW=74.55 g/mole), H₃PO₄+TBA+KCl (Solution A), H₃PO₄ 1%, 50ml TBA 30 mM, KCl 154 mM, TCA 5%, MDA standard solution (0.0627g/10ml). The procedure was as follows: 25µl of extract + 250 µl of Solution A were added to an ELISA plate, which was then incubated for 1h at 65 °C. OD was read at 532 nm. For measurements of the standard range, the extract was replaced by different concentrations of MDA.

Catalase activity assay

The catalase activity was measured by the modified method of **Cohen *et al.*(1996)**. Briefly, the cell lysate was incubated for 5 minutes in the presence of 6 mM H₂O₂ at room temperature. The reaction was then quenched by the addition of 0.75 N H₂SO₄ and 50 mM FeSO₄. The colour of the products formed was developed by the addition of potassium thiocyanate (KSCN), and the absorbance of the ferrothiocyanate product was measured at 460 nm. The standard curve of known activity of pure catalase was used to calculate the absolute values of test samples.

Glutathione S-Transferase Assay

The following solutions were used: 0.2 mM GSH, 5mM CDNB, phosphatebuffer(pH 7.4). The procedure was to add 5µl of the extract + 200 µl phosphate buffer + 20 µl GSH + 20 µl CDNB onto the ELISA plate, after which the absorbance was read at 340 nm every 30 seconds, 4 times. For the blank the same was added but the extract was replaced with phosphate buffer.

Statistical analysis

The obtained data were analysed statistically using Prism Statistical software program to calculate T-test and ANOVA.

CTAB method for DNA extraction

DNA was extracted from the liver cell, the host of the parasite by CTAB precipitation method (**Yap and Thompson, 1987**).RAPD-PCR technique was carried out using three decimer (AP1: 5'-GGAAACCCCT-3', AP2: 5'-GGAAGTCGCC-3' and AP3:5'-GTAGGCCTCA-3').The lyophilised primers were dissolved in sterile distilled water with a final concentration 100 p mole/ml for each primer. The PCR mixture contained 5X PCR buffer, 10mM dNTPS mixture, 25 mM MgCl₂, 0.75 mM bovine serum albumin (BSA) (Sigma Germany), (2U) Taq DNA polymerase, 50 pmole) arbitrary primer, template DNA (according to the concentration of the cestode extracted for genomic DNA, the mixture volume was completed to 25µl with sterile ddH₂O. Amplification was carried out in ESCD swift max thermo cycler, Singapore. Thermal cycler was programmed for 45 cycles of 1 minute at 94°C, 1 minute at 36 °C and 2 minutes at 72°C. The amplification products were analysed by electrophoresis on 2% agarose gel in TAE buffer and detected by staining with ethidium bromide. The observed bands were scored as presence or absence for each group, and then the commonality percentages between all groups were calculated based on pairwise comparison between them for a particular primer, using the formula below, where N_{xy}: the number of shared bands between x and y; and N_x and N_y: the number of bands in individuals x and y (**Lynch, 1990**): Commonality Percentage = $N_{xy}/(N_x+N_y) \times 100$.

III. Results

Microscopic examination

As shown in Fig.(1), the morphological characteristics of cysticerci recovered from the local and imported sheep under study were almost similar. Table (2) demonstrated the measurements of rostellar hooks and hook parts of cysticercus recovered from both local and imported sheep.

Biochemical Results

The determination of reduced glutathione (GSH), Malondialdehyde (MDA), Catalase enzyme activity (CAT) and glutathione S transferase, (GST) levels in liver tissues revealed a significant difference between control samples and infected ones of either imported and local sheep where the ratio of the mentioned parameters increased significantly in the infected groups compared to the respective control ones as described in Table 2.

RAPD-PCR

Out of ten tested arbitrary oligonucleotide primers, three primers of random sequences (AP1, AP2, AP3) produced recognisable fragments in RAPD-PCR and generated polymorphic DNA fingerprints of normal and infected livers for both imported and local sheep. As shown in Fig. 2, the primer AP1 produced polymorphic DNA fragments in the range of 100 to 1500 bp, most of which were reproducible with genomic DNA of liver isolates from sheep origin. A dominant fragment of 280, 800 bp was prominent in control local sheep isolates and absent in control imported sheep, while a fragment of 300 bp was prominent in infected local sheep isolates and faint in infected imported sheep. In respect with the primer, AP2 (Fig.3), polymorphic DNA fragments in the range of 100 to 1500 bp, were produced most of which were reproducible with genomic DNA of liver isolates from sheep origin. A dominant fragment of 300-550 bp was prominent in control local sheep isolates and absent in control imported sheep, while a fragment of 700-800 bp was prominent only in infected local sheep isolates and faint in infected imported sheep. A dominant fragment of 100 bp was prominent in control

imported sheep isolates and absent in infected imported sheep, while a fragment of 1200 bp was prominent only in infected imported sheep isolates and faint control imported sheep.

Fig. 4, showed the primer AP₃ producing polymorphic DNA fragments in the range of 100-1500 bp. A dominant fragment of 500-800 bp was prominent in control local sheep isolates and absent in control imported sheep, while a fragment of 400-700 bp was prominent in infected local sheep isolates and faint in control local sheep. A dominant fragment of 250-500 bp was prominent in control imported sheep isolates and absent in infected imported sheep, while fragments of 350, 900, 1000 bp were prominent in infected imported sheep isolates and faint control imported sheep. Percentage of similarity in imported sheep (SC) 49% and the proportion of the difference in imported sheep (DC) 51%, while the percentage of similarity in the local sheep (SC) 38% and the proportion of variation in the local sheep (DC) 62% (Table 5). The results showed that the infected local sheep was affected more than the imported one

IV. Discussion

Cestode larval infections in sheep lead to inflammation as these larvae penetrate and imbed in tissues. Inflammation reactions are associated by production of ROS (Egwunyenga *et al.*, 2004; Pal *et al.*, 2006; Bello *et al.*, 2000). The oxidative damage resulting from increased ROS production is destructive to cells and may lead to apoptosis and cell death (Beyer *et al.*, 1996). Furthermore, the increased oxidative stress contributes to liver damage and fibrogenesis, and also leads to increased eosinophil production; which subsequently leads to mitochondrial injury and apoptosis of eosinophils (Serradell *et al.*, 2009).

Additionally, earlier studies on malaria infection, high levels of oxidative stress markers have been observed in infected humans and rats in comparison to healthy controls (Clark and Hunt 1983; Sobolewski *et al.*, 2005; Guhaet *et al.*, 2006; Sohailet *et al.*, 2007). Several tapeworm species that cause organ damage to mammalian liver have previously been studied. These include *Taeniahydatigena*, *T.taeniaeformis*, *T. Crassiceps*, *Echinococcus granulosus* and *E. Multilocularis*. Infestations have been observed to raise ROS levels that eventually lead to an increased oxidative stress index and a decreased total antioxidant capacity (Assady *et al.*, 2011). The early stages of cyst development are usually accompanied by a concentration of eosinophils, neutrophils, and macrophages that surround the cyst (Zhang *et al.*, 2003).

In their study, Li *et al.* (2004) described the functional protein and mRNA expression, along with the localisation of a peroxiredoxin from *E. granulosus*. Similar to our study, they observed fluid-filled bladders as hydatid cysts that develop in the internal organs of hosts. They also observed that the cyst survived under oxidative stress, and in the aerobic environment conditions induced by ROS. Glutathione plays an important role in detoxification reaction in liver and in regulating the thiol-disulfide status of the cell. Also, degradation of hepatic glutathione is regulated by the efflux of reduced and oxidised glutathione into bile and sinusoidal plasma. Under oxidative stress condition, the liver exports oxidised glutathione into bile in a concentrative manner, while under basal conditions, mainly reduced glutathione is exported into bile and blood.

However, Cunha *et al.* (2012) reported impairment of the liver GSH levels content in schistosomiasis infected mice. It was suggested that the antioxidant capacity of the liver decrease as a result of this type of infection. This subsequently leads to the generation of lipid peroxides that in turn play a further central role in the pathology associated with schistosomiasis. Accordingly, Hamed (2006) stated that after parasitic infection, glutathione levels were decreased. Gharibet *et al.* (1999) explained that this decreased level of glutathione is attributed to the increased cytotoxicity with H₂O₂. The increased H₂O₂ occurred as a result of inhibition of glutathione reductase that keeps glutathione in its reduced form. In the present study, the determination of total GSH level in liver tissue revealed a significant difference between local control samples and infected ones, where glutathione ratio increased significantly in the infected groups compared to their respective control ones. Further, glutathione levels were higher in the infected local sheep than the infected imported sheep samples, at odds to (Kaplowitz, 1981).

Oxidative stress may result from increased production of free radicals due to increased MDA levels, an important lipid peroxidation marker. Generally, most parasites have to deal with the oxidative level inside their host cells. ROS and oxidative stress are the result of the aerobic metabolism that generates highly reactive metabolites of molecular oxygen in the cytosol, or in organelles such as the mitochondria or the peroxisomes (Andreyev *et al.*, 2005).

In this study an increased level of MDA was reported in infected sheep. The MDA level was also higher in the infected imported sheep samples, compared to the infected local sheep samples. In addition, Glutathione S-transferase activity in liver tissue was significantly increased in infected sheep compared to control groups. Our MDA results are in agreement with studies that have found significantly increased levels of MDA in serum of human patients with fascioliasis when compared to controls (Kaya *et al.*, 2007) and human patients with *Plasmodium falciparum* caused malaria (Egwunyenga *et al.*, 2004 and Ismail & Al-Kennany, 2012).

Catalase (CAT) is a clue of the antioxidant defence system. It plays an important role in keeping homeostasis and protection against oxidative damage through the remove of the toxic free radicals (EI-Shenawy *et al.*, 2008; Jia *et al.*, 2009).

The present data demonstrated that the activity of the liver tissue CAT of the infected sheep significantly increased as compared to control group. This is in agreement with the report of Kolodziejczyk *et al.* (2005) in their study on rats experimentally infected with *Fasciola* where they stated that catalase (CAT) activity in liver increased simultaneously with enhancement of lipid peroxidation processes as evidenced by the elevation of malondialdehyde (MDA). On the other hand, Rizk *et al.* (2012) recorded a reduced level in catalase activity in schistosomiasis infected mice and they attributed this to utilization of catalase in scavenging free radicals' overload, which generated during schistosomiasis infection

Severe discrepancies in the reactive oxygen species (ROS) level can induce oxidative modifications in the indispensable cellular macromolecules such as DNA, proteins, and lipids (Franco R. *et al.* 2007), ultimately leading to cell death. In normal aerobic cells, reactive oxygen species (ROS) are generated as a by-product of conventional mitochondrial activity. If uncontrolled properly, ROS can result in severe damage to certain macromolecules, especially the DNA. Therefore, we tried to trace the DNA changes in control and infected sheep through RAPD-PCR assay.

In the present study, RAPD-PCR procedures were carried out using three primers; AP1, AP2 and AP₃. All primer produced polymorphic DNA fragments (ranging from 100- 1500 bp), most of which were reproducible with genomic DNA of liver isolated from sheep. Certain fragments were prominently observed in control local and absent in control imported sheep. On the other hand, specific bands were prominent in infected local sheep and faint in infected imported sheep and vice versa. .

Percentage of similarity was measured between the different sheep samples; showing that the infected local sheep was more affected than the imported samples. In accordance with Li *et al.* (2004), major biological macromolecules may thus be damaged caused by the oxidative stress of the highly unstable ROS; leading to strand breaks, DNA base modifications, lipid peroxidation and protein oxidation. This was investigated by Sharma *et al.* (2012) where they stated that The ZnO NPs induced oxidative stress led to DNA damage and apoptosis in the mice liver. They used comet assay, which revealed a significant increase in DNA lesions in liver indicating oxidative stress as the cause of DNA damage.

The results of the present study proved that RAPD technique is a useful tool for detection of DNA damage induced by parasitic infection in sheep. Similarly, Castano and Becerril (2004) used RAPD to assess DNA damage of the RTG-2 fish cell line after short- and long-term exposure to benzo (a) pyrene. By comparing DNA fingerprints from control and exposed cells, an increase in the instability in the DNA fingerprint of exposed cells over a time- and concentration-dependent manner was observed. .

Also, Symonds *et al.* (2008) found that Methoxychlor (MXC) induce oxidative stress damage of DNA in breast cells and mouse ovarian follicles. Also, Methoxychlor produce adverse effects on reproduction that correlate with altered DNA methylation Patterns in the germ line of rats. In the same mannaer, Guidaet *al.* (2010) assessed DNA Damage by RAPD in *Paracentrotus lividus* Embryos Exposed to Amniotic Fluid from Residents Living Close to Waste Landfill Sites and they proved the ability of RAPD-PCR to detect and isolate DNA sequences representing genetic alterations induced in *P. lividus* embryos.

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Table 1: Rostellar hooks and hook parts measurements of *T. hydatigena* cysticercus of imported (I) and local (L) sheep

No	Sample source	Hooks No.	Hook Length	Large hook parts	Small hook parts
		Large/Small	Large/Small	Blade/Guard/Handle	Blade/Guard/Handle
1	Imported sheep	15/15	0.17/0.11	0.08/0.03/0.09	0.06/0.02/0.05
2		16/16	0.22/0.08	0.14/0.03/0.08	0.05/0.02/0.03
3		20/20	0.19/0.13	0.1/0.04/0.09	0.09/0.03/0.04
4		13/13	0.17/0.11	0.08/0.03/0.09	0.06/0.03/0.05
5		13/13	0.18/0.1	0.08/0.02/0.1	0.04/0.02/0.06
1	Local sheep	14/14	0.17/0.17	0.11/0.03/0.06	0.06/0.02/0.05
2		16/16	0.16/0.1	0.1/0.02/0.06-7	0.07/0.01/0.03
3		14/14	0.21/0.1	0.11/0.03/0.1	0.06/0.02/0.04
4		14/14	0.2/0.12	0.11/0.05/0.09	0.07/0.04/0.05
5		20/20	0.2/0.12	0.1/0.03/0.1	0.06/0.02/0.06

Table 2: oxidative stress markers levels in hepatic tissues Healthy and infected sheep (mmoles/mg protein)

Group	(GSH) Average±SD	(MDA) Average±SD	(CAT) Average±SD	(GST) Average±SD
CI	0.79±0.17 ^{a,d}	0.028±0.0096 ^{a,d}	0.423±0.050 ^{a,c}	0.011±0.0014 ^{a,c}
CL	1.52±0.38 ^{b,d}	0.027±0.002 ^{b,d}	0.488±0.050 ^{b,c}	0.0060±0.0024 ^{b,c}
II	1.45±0.27 ^{a,c}	0.095±0.003 ^{a,c}	0.66±0.0053 ^{a,d}	0.022±0.0024 ^{a,d}
IL	2.96±0.18 ^{b,c}	0.043±0.009 ^{b,c}	0.66±0.0048 ^{b,d}	0.0083±0.0018 ^{b,d}

CI: control healthy group of imported sheep; CL: control healthy group of local sheep; II: infected group of imported sheep; IL: infected group of local sheep

Table (3): Number of monomorphic (mono) and polymorphic (Poly) bands among 5 isolates in three primers:

Primer	Imported sheep		Local sheep		Imported and local sheep	
	Mono-	Polym-	Mono-	Poly-	Mono-	Polym-
AP ₁	4	0	2	3	2	0
AP ₂	6	2	1	5	1	0
AP ₃	1	2	1	4	0	1

Table (4): Average number of shared bands (SB) among individuals from the same and different isolates, including similarity coefficient (SC) and genetic differences (GD) of imported sheep and local sheep:

Isolates	SB	SC%	GD%
CI&II	183	49%	51%
CL&IL	107	38%	62%

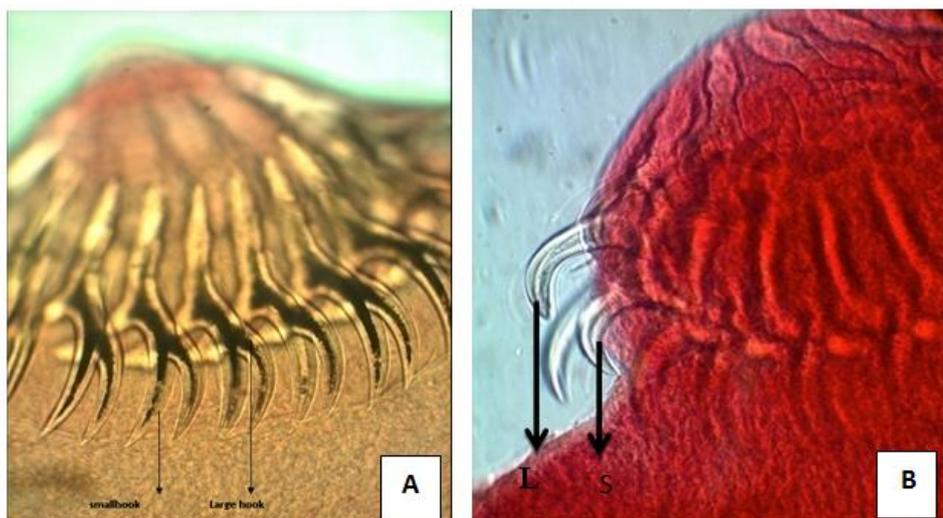


Fig. 1: Photomicrographs of the protoscolex of *Taeniahydatigena* recovered from local (A) and imported (B) sheep showing the rostellum carrying regular, alternate hammer shaped large (L) and small (S) hooks.

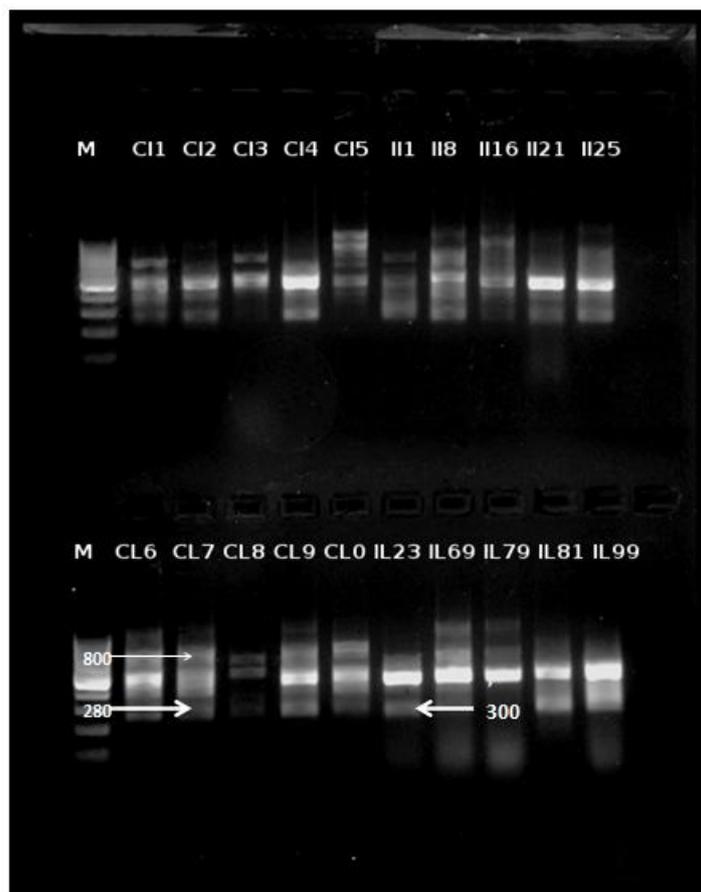


Fig. 2: 2% agarose gel electrophoresis of RAPD-PCR using primer AP1. M: 1500~75 bp DNA ladder. CI/II:Control/infected-imported sheep. CL/IL: control/infected local sheep.



Fig.3: 2% agarose gel electrophoresis of RAPD-PCR using primer AP2 M: 1500~75 bp DNA ladder. CI/II:control /infected imported sheep. CL/IL: control /infected local sheep.

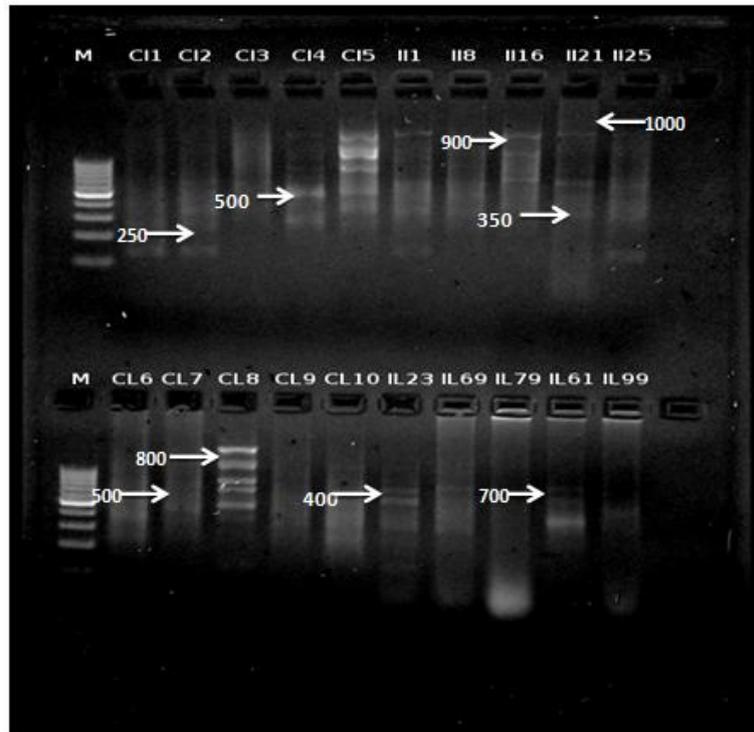


Figure 4:-2 % agarose gel electrophoresis of RAPD-PCR using primer AP3. M: 1500~75 bp DNA ladder. CI/II: control /infected imported sheep, CL/IL: control/infected local sheep.