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A Molecular and Microscopically Studies of *Calicophoron Microbothrium* (Paramphistomatidae)

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ABSTRACT

Background: Paramphistomiasis is a parasitic disease of livestock animals and humans, which causes heavy economic black lashes especially in countries with advanced animal industry

Aim of Study: the current study aimed to add more information about *Calicophoron microbothrium* (*C. microbothrium)* and clarify its biological role and how its miracidia infecte the molluscan intermediate host. In addition, a brief description to *Bullins truncates*; the morphological, structural and chronological characteristics of the various intermolluscan stages of the parasite are studied in detail. Moreover, the present work showed the effective role of physical parameters (light, temperature, salinity and gas-phase (aerobic versus anaerobic)) on egg development and hatching and the biological activities of cercaria and metacercaria. Beside these routine techniques, PCR also was used as more advanced and accurate diagnostic technique based on the detection of nucleic acid. Where, 34 larvae and adult worms of *Calicophoron microbothrium* were isolated from naturally infected buffaloes. The results of the present study will facilitate the identification of this despise secular group of digeneans although its bad effect not only affect animal industry but also human health. Furthermore, the current research clears the weak points in its life cycle to aperient settling this parasite.

Keywords: *Calicophoron microbothrium, Bullins truncates*, paramphistome, biology, histology, Mas–PCR technique.

INTRODUCTION

Paramphistomes are spreading worldwide, especially in the warmer regions such as: Australia, Africa and India, and mainly infect cattle, goats and sheep. In addition, there are certain species of paramphistomes that infect human it has medical importance beside their economic one. *Calicophoron microbothrium* (*Paramphistomum microbothrium*) is one of two of these parasites in Egypt as reported by **Ashour** [(1)]; the other species was *Calicophoron gregarius.* They were the most important flukes identified in domestic Egyptian ruminants.

This *C. microbothrium* has a life cycle similar to that of the gastrointestinal trematodes. Where, the adult flukes reside in the host's rumen and reticulum; their eggs are passed through host's faeces. When eggs reach water the miracidia hatch within 12-16 days and penetrate suitable intermediate host. The parasite has a number of generations of rediae before the production of free swimming cercariae that are changed to metacercariae after encysting on water plants; these plants become the source of the ruminant food causing the infection and beginning a new cycle [(2)]. Seriously, these parasites may survive up to years, so that they become a virtually constant infestation source for numerous snail generations. As a result to that, the intermediate host with its ability of widespread, surviving for several months and shedding numerous cercariae, and then so dangerous threaten to the domestic animals [(3)].

The paramphistomes are conical or cylindrical digenean with thick bodies. They are distinguished from other flukes by the possession of a posteriorly located acetabulum. The most familiar species are parasites of domesticated livestock. It has been considered, for a long time, that paramphistomes are completely non-injurious to their vertebrate hosts, but this view has been challenged by many authors [(4-7)]. Where, an acute infection of calves and sheep had been caused by the immature conical flukes in the small intestine, particularly in advanced cattle raising regions causing elevation of mortality rate in sheep about 30% as reported by **Chauhan *et al.*** [(8)] and 21%-37.4% in cattle by **Pande** [(9)]. In addition, **Katlyar** and **Varsheney** [(10)] recorded an average percentage morbidity and mortality 41.24% and 57.62% in sheep and 68.59% and 75.53% in goats respectively. Moreover, **Horak** [(6)] recorded a loss of 11.4 kg of sheep's weight within 52 days and 27.3 kg in bovines after 140 days, while an uninfected bovine gained 50.9 kg during the same period.

The clinical signs of acute paramphistomiasis in sheep, goats and cattle have been represented by

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restless and progressive decrease in appetite developed to complete anorexia, small quantities of water are taken and the animals might stand with their muzzles in water for a long time; a condition known as polydipsia. Diarrhoea developed 2-4 weeks after infection, with fetid faeces and rectal hemorrhage. Submandibular oedema (bottle jaw) has been noted in a number of outbreaks. Oedema of lungs, hydrothorax, hydropsyeasdium and ascites were reported by [**Baldrey** [(11)], **Walker** [(12)], **Chauhan *et al.*** [(8)], **Pande** [(9)], **Haji** [(13)], **Bawa** [(14)], **Boray** [(15)], **Varma** [(16)], **Horak** and **Clank** [(17)], **Katiyar** and **Vershney** [(18)] and **Horak** [(6)]].

About how the parasite causing its disorder effects in animals, **Horak** [(7)] stated that immature paramphistomes might penetrate the intestinal wall to just below the serosa and can be seen from the peritoneal side of the intestine. On rare occasions, the parasites perforated the intestine and were found in the abdominal cavity. While, **Dinnik** [(4)] and **Horak** [(6)] reported that many cattle and sheep were infected with adult paramphistomes and the infection was acquired by ingestion of few numbers of metacercariae on one or several occasions, didn't cause an obvious harm to the host. The ingested metacercarine mature rapidly and serve as a source of infection for successive generations of snails. These paramphistomes can survive for some years, where the source of infection is virtually consistent.

The average daily egg produced by a single *C. microbothrium* in infected sheep was estimated about 75 eggs [(6)]. Although this was not a large output but the large number of parasites was usually found in host with large number according to **Boray** [(19)] who found 60.000 worms or excess of *C. ichikawai*/ 3-8 naturally infected sheep and **Dinnik** [(4)] returned that to long life span of *C. microbothrium* in cattle which maintains its egg production for many years, that appearing the severity of this parasite.

Surprising, it was possible that some species of paramphistomes which are parasites of wild ruminants were occasionally encountered in domestic ruminants if wild ruminants occasionally infect domestic ruminants' places and stocks live closely together [(20&21)].

Notably, there was asymptomatic in paramphistomes' natural hosts such as pigs and monkeys, while in human causes serious health problems like diarrhea, fever, abdominal pain, colic, and an increased mucous production. In addition, in extreme situations this disease leads to a number of mortality among children like happened in Assam, India [(22)].

Although the dangerous effects of this parasite on humans and economic animals, there were limited number of papers has been studied the paramphistomes in Egypt. **Ezzat** [(23)] described specimens from *Gazella dorcas* which were identified as *Paramphistomum cervi*. **Tadros** [(24)] reported the presence of paramphistomes in cows, buffaloes, sheep and camels in Shebin El-Kanater district in the Nile Delta, but he did not designate these to known genera or species. **Abdel-Ghani** [(25&26)] described specimens, which were identified as *P. cervi* but his description was not based on anatomical and histological features which were considered necessary for the specific identification of paramphistomes. Also, he described briefly the eggs and miracidia of these flukes and conducted some experiments to determine the susceptibility of small laboratory animals to infection. **Ashour** [(1)] gave the first detailed morphological, anatomical and histological account of paramphistomes in Egypt. He recorded two species under the genus *Paramphistomum*, namely, *C.* (= *Calicophoron*) *microbothrium* and *C. khalili* as well as one species in the genus *Carmyerus*, *C. gregarius*. Moreover, he conducted comparative morphological and experimental studies on the eggs and miracadia of both *P. microbothrium* and *P. gregarious*. **Elkabbany** [(27)] gave more information about scanning and transmission electron microscope of two species of paramphistomes, *C. microbothrium* and *C. gregarious*.

In addition to the previous causes, the difficulty of paramphistomes' identification morphologically and histologically leads to find new method easier. Therefore, the current study used the PCR based techniques beside the routine techniques which should be a suitable tool because it was providing rDNA ITS2 sequences that have proven as a suitable marker for identification of paramphistomes species [(28)].

MATERIAL AND METHODS

1 Field studies:

1.1 Collection of paramphistomes:

Mature specimens of paramphistomes were collected from cows, buffaloes, sheep, and goats, which slaughtered at Sharkiya and Mit Ghamre Abattoirs. The flukes were found attached by their strong acetabula to the inner surfaces of the rumen and the reticulum of their hosts. They appear in groups as pinkish to red

flesh-likes patches in case of *Calicophoron*; however, Few flukes were seen free in the lumen of the stomach.

After the removal of the flukes from the stomach, the flukes being attached to each other, therefore, they were put in normal saline solution (0.7% NaCl). Where, saline make the fluke's exhibit slow movement, in which the body becomes narrower, elongated and the anterior end of the body moves in different directions, while the posterior end is usually fixed to another fluke. Then all these samples were stored in 95% ethanol.

1.2 Morphological identification:

Some fresh flukes were pressed between two glass slides and fixed in 70% alcohol for two days. The relaxed flukes were stained for identification according to **Eduardo** technique [29]. All specimens were examined morphologically and confirmed as paramphistomes before they included in molecular study.

1.3 DNA extraction:

Genomic DNA collected from a small part of adult flukes was extracted by the alkaline-lysis (Hot-SHOT) method [30]. Mass-PCR technique for detection of *C. microbothrium* was carried out at molecular unit of Microbiology Department, Faculty of Medicine Zagazig University.

1.4 Molecular analysis

The ITS2 (internal transcribed spacer region 2) region was amplified using the primers GA1 [5_-AGA ACA TCG ACA TCT TGA AC-3_] [31] and BD2 [5_-TAT GCT TAA ATT CAG CGG GT- 3_] [32]. Polymerase chain reaction (PCR) cycles were performed on Eppendorf Mastercycler epigradient machines. After the thermocycler process was finished, PCR products were purified using PCR Microcon columns and both strands were sequenced using an Applied Biosystems 3100 automated sequencer.

1.5 Agarose gel electrophoresis:

Amplification products of *C. microbothrium* adult flukes were visualized by electrophoresis on 1.5% agarose agar and staining by 0.5 µg/ml ethidium bromide in the running buffer procedure of **Viljoen *et al*.** [33]**.**

2 Laboratory studies:

The other part of the current study was carried out in the Labe. Amounts of *Calicophoron*'s mature parasites and eggs were collected from infected sheep. In addition, snails those were controlled under the stereomicroscope for determination of natural infection.

2.1 Preparation of tissue sections for histological studies:

Snail's tissues for histological studies, using haematoxylin, eosin and toluidine blue as the method of **Krichesky** [34].

2.2 Follow up the life cycle of *C. microbothrium*:

Using both morphological and histological examination to all stages of the parasite life cycle in the intermediate host and adult flocks.

RESULTS

Examination of *C. microbothrium*'s eggs revealed they are oval operculated, smooth-surfaced and white-grayish in color; their length 130-170 µm and width 70-100 µm (Fig 1). In the central of the egg, embryo was seen in an early stage and surrounded by yolk cells (Figs 2 & 3). While, Figure 4 was showed the escaping process of miracidium and showing operculum and egg opening. Notably, the hatching of *C. microbothrium*'s eggs occurred more on the same periods in variable water mediums at the same temperature.

C. microbothrium's miracidium shape like a torpedo; it was covered with cilia and carried on eye spots (Fig 5). The average size of miracidium was measured about 199-270 x 50-60 µm. Figs 6 & 7 showed miracidium penetrated the *Bulinus truncatus* snail through the mantle cavity.

By following up the infection stages of snail by miracidium, using dissection of snail at different periods to detect the stages of Sporocyst, it was found that Sporocyst was sacculer, elongated and curved in shape and measured 200 µm x 100 µm (Fig. 8, 9, 10 & 11). Interestingly, Sporocysts were detected mainly around the intestines and in mantle tissue of snails.

While**,** rediae were opaque, slightly curved in shape and had a limited cavity as observed in Fig 12. In the young rediae the pharynx and the intestine were visible and they contain embryo balls from which cercariae or daughter rediae would develop (Figs 12 & 13). Rediae were detected around the intestines, hepato-pancreas and in the mantle tissue of *Bulinus truncatus*. The developing rediae showed great variations in size and the mature rediae measured 700-1170 µm in length and 130-260 µm in width (Fig. 14).

Otherwise, immature cercariae were liberated from rediae before their complete development. In the young cercariae the body was small, eye spots were prominent and

pigment and the tail was short and wide rather than long (Figs. 15, 16, 17 & 18). But the mature cercariae were large, active, and dark pigmented and their body was 500-550 µm long and 200-400 wide; while their tail was 550-560 long and 80-90 µm wide (Figs 18, 19, 20, 21 & 22).

Notably, it was seen that light was a great factors in shedding cercariae, infected snail kept in the dark started shedding cercariae again after being exposed to light. Cercariae were collected from the snails only one day in a week in order to regulate metacercarial age conformity and to obtain then in high numbers.

Under the light source and in the presence of vegetation in the water most cercariae encyst on the vegetation. During the encystment cercariae attaches with its ventral surface, and the material for cystation being to secrete from the pores all over the body (Figs 23 & 24). Metacercaria was in the form of a half sphere and was surrounded with a thick layer of a cyst wall. These metacercariae were measured 240-260 µm in diameter (Figs 25, 26 & 27).

The sensitivity of polymerase chain reaction (PCR) was measured using various number of lysate cells of *C. microbothrium*. After 22 cycles of PCR as little as one copy of the target gene was detracted by specific primer on agarose gel electrophoresis (Fig. 28). Each primer (P_1 and P_2) amplified several DNA fragments that were polymorphic the species. The results which obtained with primer 1 and 2 produced characteristic intense band patterns of C. *microbothrium* (769 bp) (Fig. 28).

DISCUSSION

It is well known that the intermediate host of *C. microbothrium* varies according to the geographical regions; however, many types of snails play the main role as intermediate host [25 & 35-41], *Bulinus truncates* were recorded as an intermediate host for heavy infestation of the snails with various paramphistomes is reported mainly in the late summer and autumn months and the incidence varies between 3 to 75% in the infected areas [36-38 & 42-44]. In the present study naturally infected *Bulinus truncatus* were found at each month between April and November too.

Otherwise, it was noted that many factors affect the development of Paramphistome eggs such as temperature and light; but the effect of temperature rather than that of light [45-47]. Similar results were obtained from the current study, where the development of *C. microbothrium* eggs in various mediums and temperatures did not show any variation; while, light was found to be the most effective influence factor for hatching.

On the other hand, it was noted that tap water is a good medium than distilled water and saline solution (0.9%) for several of *Paramphistomum* miracidia and some miracidia of *Paramphistomum* species kept their viability longer in lakes and pools compared with other media [46-48]. The previous results supported our finding that newly emerged miracidia swam more active in distilled water and spring water.

Otherwise, it was recorded that too many miracidia had a negative effect on the experimental infection of snails and only the young or medium sized snails were suitable for infection [47&49]. During the current experiment, it was observed that the infection rate increased with the number of miracidia used; but contrast to this, it was seen that the large proportions of miracidia had a negative effect. Concerning snail size, it was seen that the infection rate among the small and middle sized *Bulinus truncatus* is elevated their mortality rate is increased too.

Generally, it was accepted that the miracidia lost their cilia during the penetration and entered the snail host tissues as a young sporocyst. On the observation of miracidial penetration into the snails, both mechanical and enzymatic effects together play a role [46-47]. The transformation of the miracidium into a sporocyst is a gradual process and no clear line can be drawn between the invading miracidium and young sporocyst [50]. In the present study persistence of cilia of the anterior part of the miracidium was also observed, 1-2 hours after the infection of snails.

Available information on the development of *C. microbothrium* in the snail is very limited [46]. **Kıselev** [51] reported that, in some Paramphistomum species, the occurrence of daughter rediae were recorded only in autumn months. Moreover, the production of redia was less in number compared to the regular production of cercariae and they were observed after the 40th days of infection yet. The current experiment showed the daughter rediae in *Bulinus truncatus* not only in autumn months but also in different months of the year. Interestingly, in the current research, it is possible to detect the rediae which caring daughter rediae from the external lateral parts of the mantle tissue of *Bulinus truncatus* before its dissection on the examination under the stereomicroscope. Notably, the location of rediae in the snail tissue was an important factor

for the production of daughter rediae; where, daughter rediae developed mainly from the rediae developed in the external circle of the snail mantle tissue.

It has been recorded that the light was the most important factor for cercarial shedding of infected snails and the snails could continue to shed about 1-10 months [46-47& 52]. We obtained not only similar results but also saw the infected *Bulinus truncatus* continued to shed cercariae in the laboratory for more than 13 months; some snails became free from infection after 11 months.

Paramphistomum cercariae are usually encysted on the vegetation and other subjects in the water and, they can stay viable for about 5 months when they are persevered in the refrigerator [25, 35 & 46]. In present study encystations occurred on the plastic sheet which was placed in the Petri dish and especially around its folds. It was also observed that one fifth of the metacercariae kept among wet filter paper in the refrigerator at 4°C kept their viability for about 6 months, while most of them started death after the second month.

Furthermore, in current study it was determined that the prepatent period of infection was 102-142 days and the infection rate was 19.6-77% in lambs. There were finding of **Abdel Ghani** [26] and **Kraneburg** and **Boch** [53] in the *C. cervi* supported the current results, where they reported that, infection prepatent period was 129 days in goats, 103-115 days in cattle and sheep and the infection rate was 45.82% in goats, 40.6% in sheep and 44.8% in cattle.

In the recent years, PCR was established as valuable tool for routine diagnosis of infectious diseases. It has been widely used as an *in vitro* method for detection of specific DNA from minute amounts of starting material. The assay did not require radioactivity, no laborious hybridizations and the procedure can be performed in one day [54&55]. **Samn** [56] reported that PCR could be used for identifying the genetic finger prints of species of paramphistome. **Luton *et al.*** [32] and **Anderson** and **Barker** [31] compared the sensitivity of many PCR primer sets for detection of *C. microbothrium*.

In PCR test was carried out by the extracted DNA from 34 worms of *C. microbothrium.* This led to doubling and finding the genetic sequence by using P_1 and P_2 the partial weight of the gene is estimated by 0.90 k Dalton. The extracted DNA from *C. microbothrium* was utilized by the DNA extraction techniques [31&32]. The purity and concentration of the DNA has been measured by utilizing the spectral system that is used in measuring the ultraviolet rays ranging from 300-350 Angstrom for measuring the DNA this agreed with **Dubs** and **Dhlaminiz** [57]. Each target gene was amplified by PCR or Mass–nested PCR with serially diluted DNA extracted from purified *C. microbothrium*. The target genes included *C. microbothrium* wall protein, small subunit ribosomal RNA (SSU rRNA) and random amplified polymorphic DNA. The present study agreed with **Lotfy *et al.*** [55] where the second-round amplification using 769 bp-primer of the target gene showed that the nested primer set specific for the gene proved to be the most sensitive one compared to the other primer sets tested and would therefore be useful for the detection of *C. microbothrium*. They added that the most sensitive Mass–PCR target gene and the primers cowpnest-F1 and cowpnest-R2 could detect as low as a single *C. microbothrium*. This highly sensitive Mass–PCR method will be helpful for facilitating the detection of Paramphistomes in specimens with a low number of worms and could be a valuable tool for detection of worm in environmental and clinical samples.

After data from the present figures were comparing diagrammatic representation banding patterns of the species *C. microbothrium* obtained with P_1 (primer) and P_2 (each of them yields clearly banding pattern for the species investigated), it was agreed with the report of **Lotfy *et al.*** [55]. Notably, **Acuna-Soto *et al.*** [58] and **Aguirre *et al.*** [59] were used the differences in the highly repetitive sequences in the non-coding region of rDNA whereas **Novati *et al.*** [60] and **Troll *et al.*** [61] based their test on the 16s rRNA gene of other paramphistomes species.

Finally, the results of DNA and PCR indicated that the use of the genetic techniques and the molecular biology have a great importance in discovering and identifying the genetic finger prints of the *C. microbothrium* that will help those who the specialized in this field in future. In addition, the current study gives general information on *C. microbothrium* life cycle and cleared points of weakness and strength that facilitated methods of elimination of this parasite.

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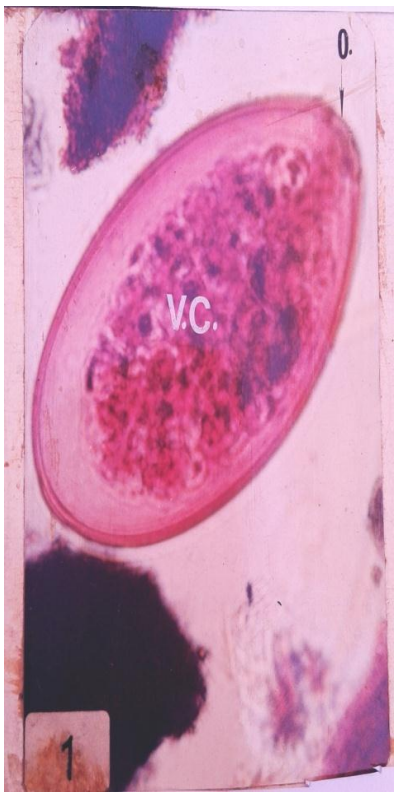

Fig (1): Photomicrograph of permanent preparation of egg stained with aceto-carmine, showing the operculum (O) and vitelline cells (V.C.). X:600

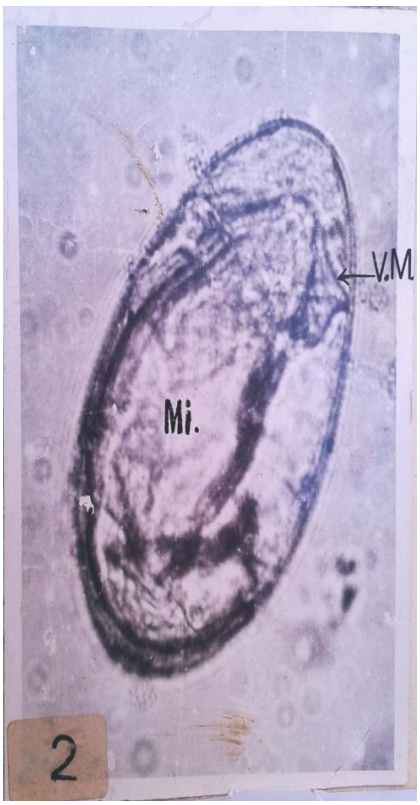

Fig (2): Photomicrograph of Egg with fully formed miracidium (Mi) during rupture of the vitelline membrane (V.M.). X:500

Fig (3): Photomicrograph of The mira-cidium (Mi) leaving the shell. X:400

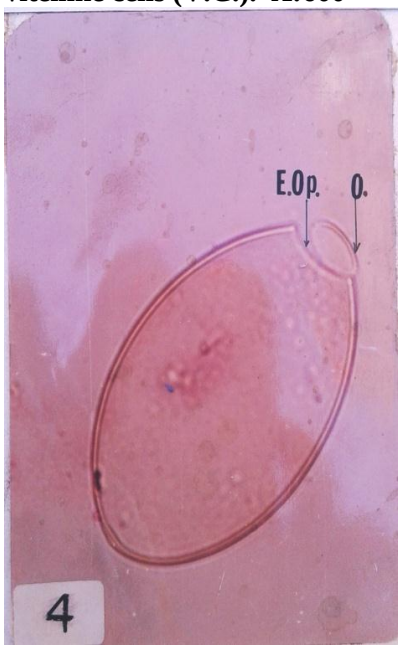

Fig (4): Photomicrograph of an empty egg after the miracidium has escaped showing operculum (O) and egg opening (E.Op). X:500

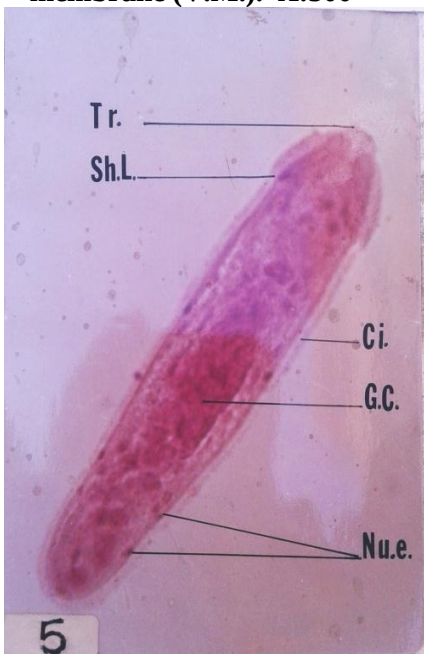

Fig (5): Photomicrograph of perma-nent preparation of the miracidium stained by aceto-carmine, showing the terebratorium (Tr.), the shoulder line (Sh.L), cilia (Ci), and nuclei of the epidermal cells (Nu.E) and germinal cells (G.C). X:400

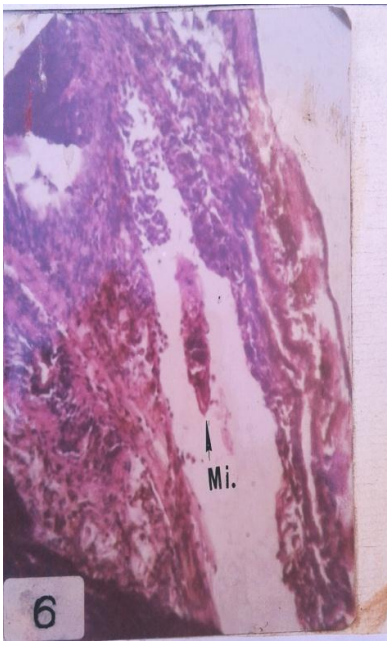

Fig (6): Photomicrograph of histolo-gical section through the mantle cavity of Bulinus truncatus showing (Mi) 30-60 minutes after penetration. X:270

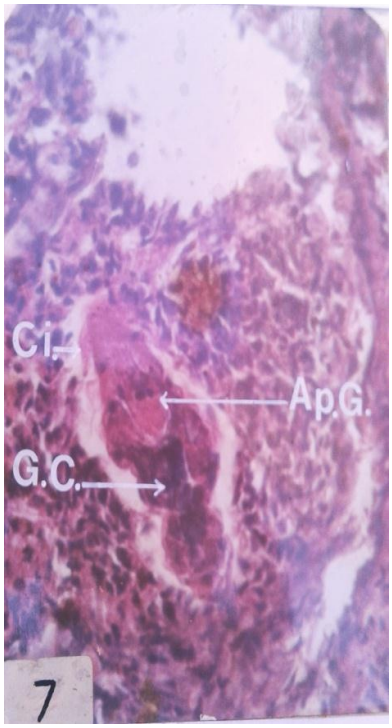

Fig (7): Photomicrograph of histo-logical section through Bulinus truncatus six hours after penetra-tion, showing the (Mi) closely lodged within the tissue, (Ci), apical gland (Ap. G.) and germ cells (G.C.). X:380

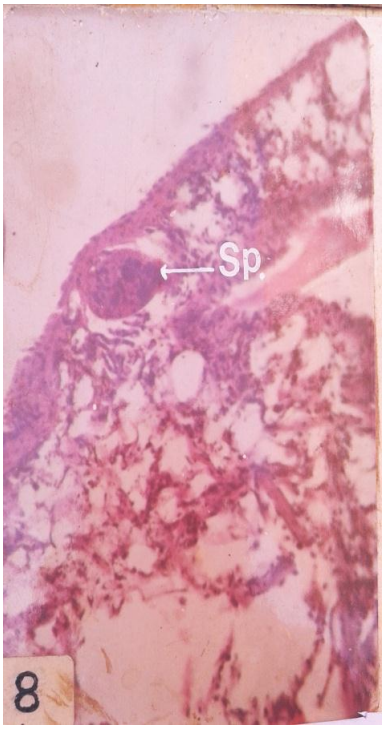

Fig (8): Photomicrograph of section through the mantle showing the young sporocyst (Sp.) 24 hours post-infection. X:400

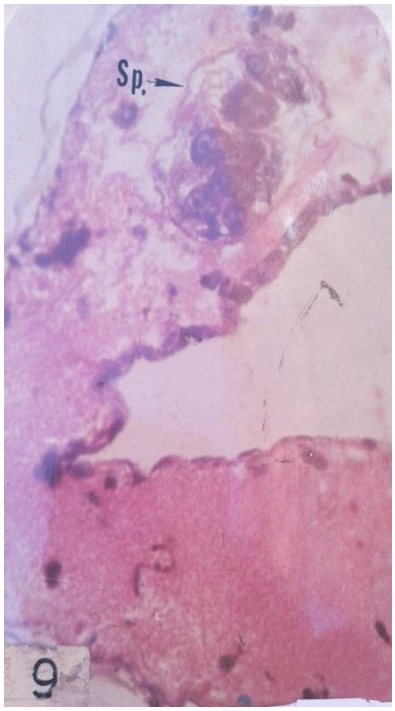

Fig (9): Photomicrograph of section through the hemolymph space showing the young (Sp.) 72 hours post-infect ion, germinal cell in mitotic status. X:400

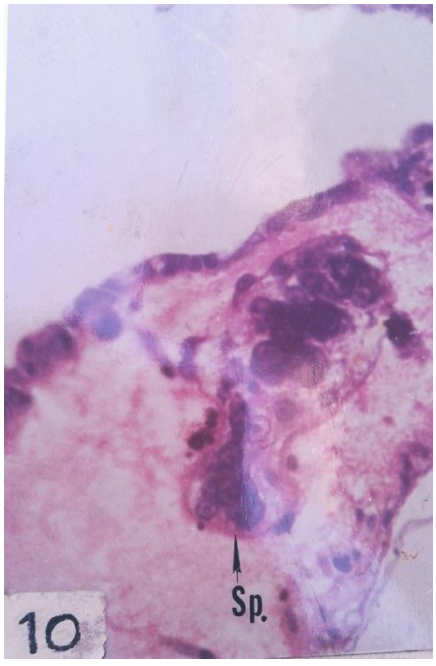

Fig (10): Photomicrograph of section through the hemolymph space showing the young (Sp.) 4 days post-infection. X:400

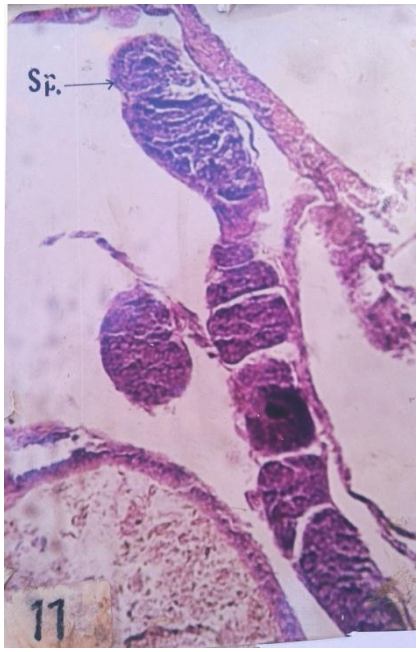

Fig (11): Photomicrograph of sec-tion through the hemolymph space showing part of the mature (Sp.) 9-10 days post-infection. X:250

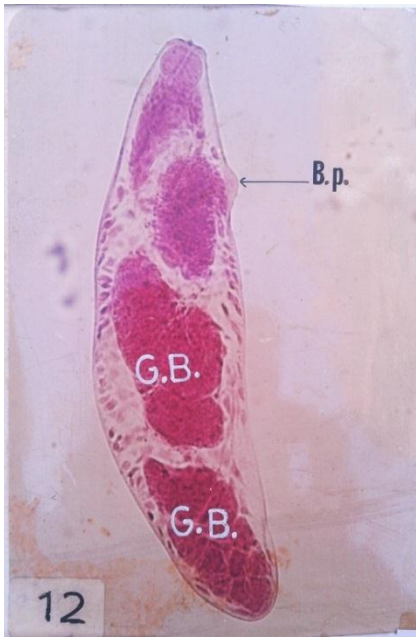

Fig (12): Photomicrograph of mature radia showing the birth pore (B.P.) and the distribution of the germinal balls. X:325

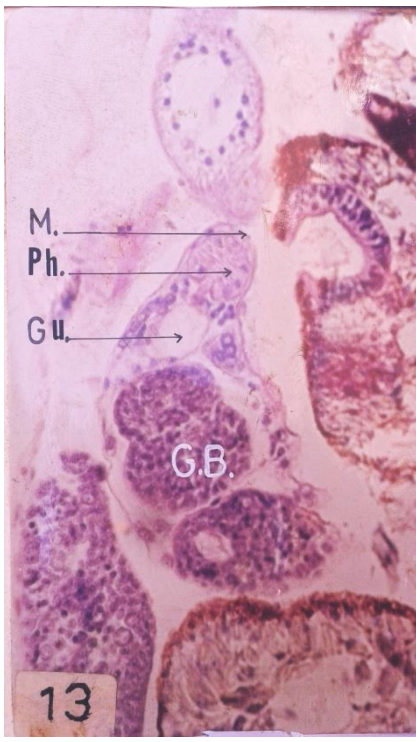

Fig (13): Photomicrograph of section of the mature mother radia showing the mouth (M), pharynx (P), gut (Gu) and (G.B.). X:450

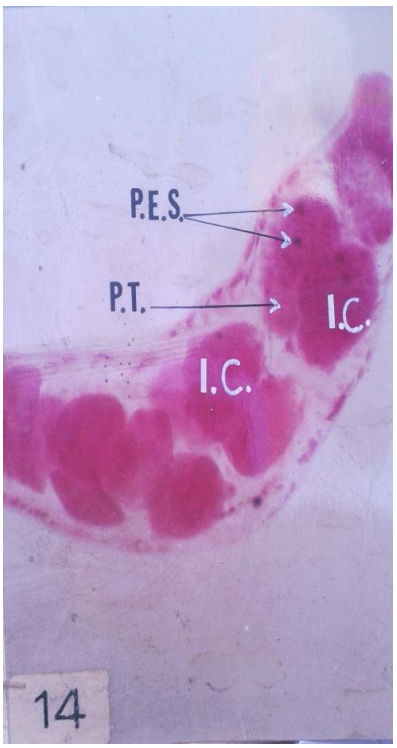

Fig (14): Photomicrograph of inter-radial stage of immature cercaria (I.C.) representing stage II of the cercarial development showing the primordia of the eye spots (P.E.S.) and primordial of the tail (P.T.). X:170

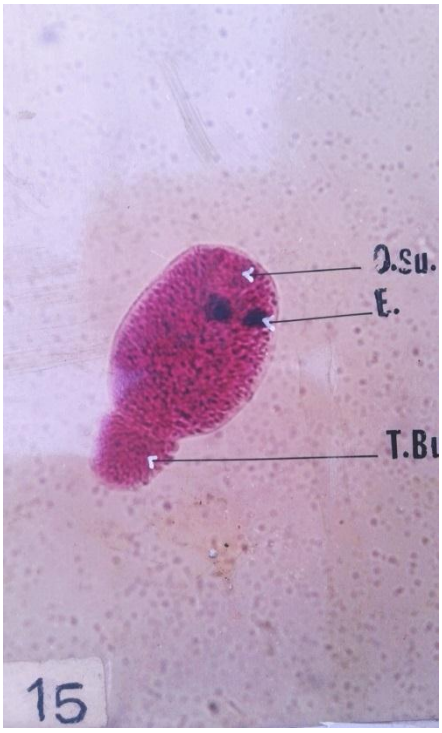

Fig (15): Photomicrograph of extra-radial stage of immature cercaria representing stage III of cercarial development, showing the primordial of oral sucker (O.Su.), eye spots (E.S.) and tail bud (T.Bu.). X:170

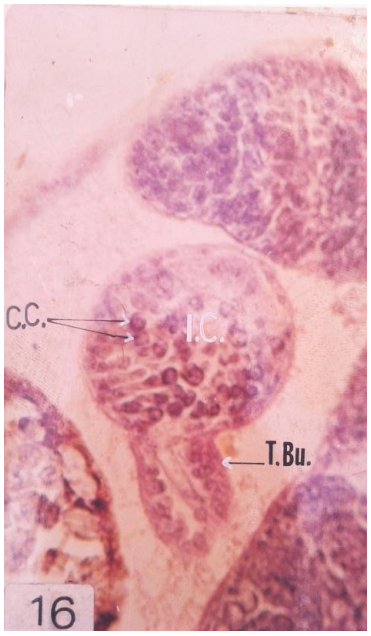

Fig (16): Photomicrograph of sec-tion through the infected snail showing the internal structure of immature cercaria (I.C.) cystogenous cells (C.C.) and T.Bu. X:150

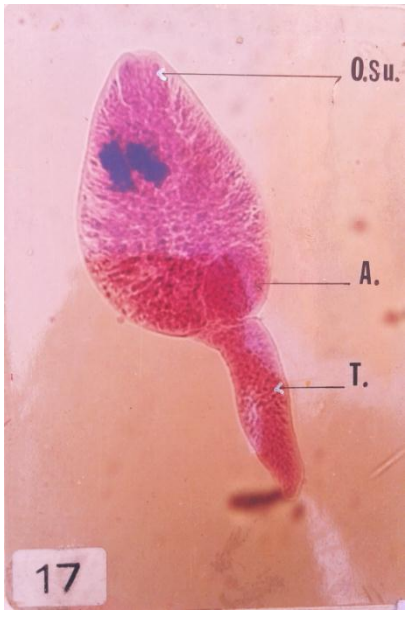

Fig (17): Photomicrograph of extra-radial stage of immature cercaria representing stage VII of cercarial development, showing the oral sucker (ACE), acetabulum (A) and Tail (T). X:170

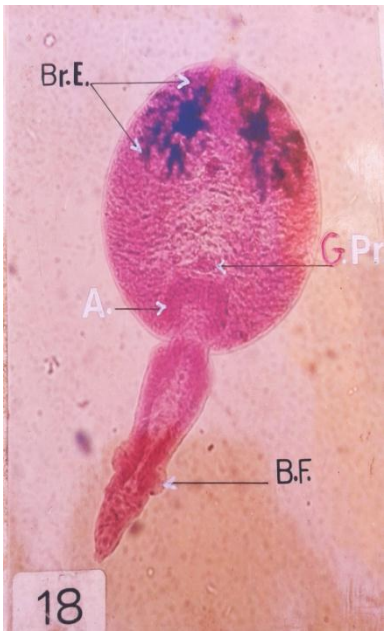

Fig (18): Photomicrograph of extra-radial stage of immature cercaria representing stage IX of cercarial development, showing the branches of eye spots-pigment (Br.E), the bud of the tail fin (B.F.). X:180

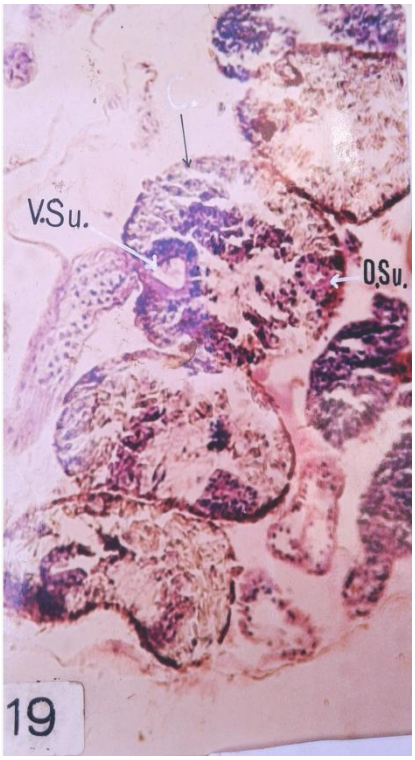

Fig (19): Photomicrograph of section through infected snail showing the mature cercariae with oral sucker (O.Su.) and ventral sucker (V.Su.), eye spots (E.S.). X:350

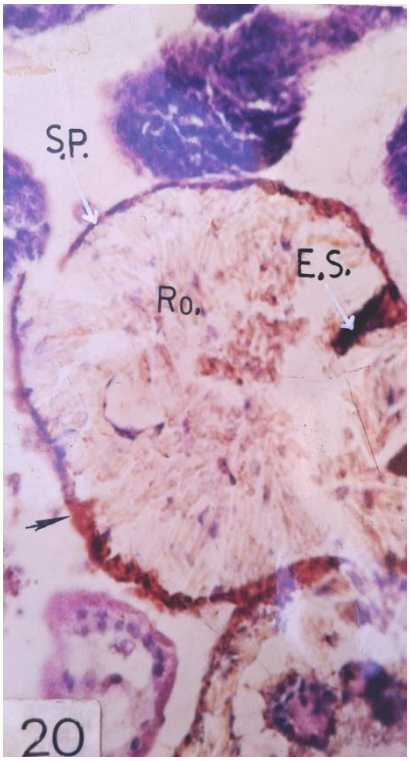

Fig (20): Photomicrograph of section through the body of mature cercariae showing the superficial pigment (S.P.) of tegument rods (RO.) and eye spots (E.S.). X:580

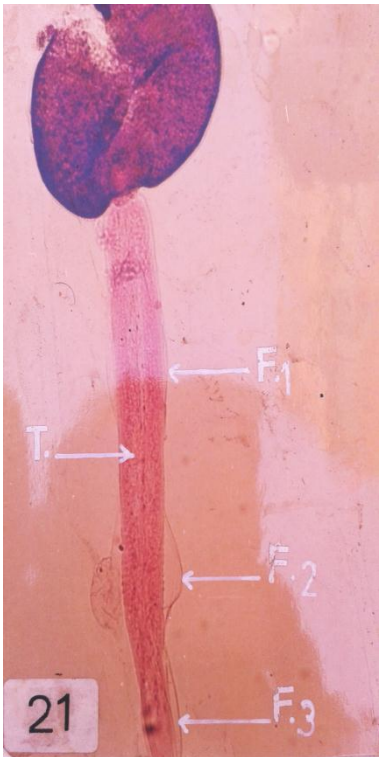

Fig (21): Photomicrograph of mature cercariae showing the tail (T) and mucoid tail fins (F1, F2, and F3). X:120

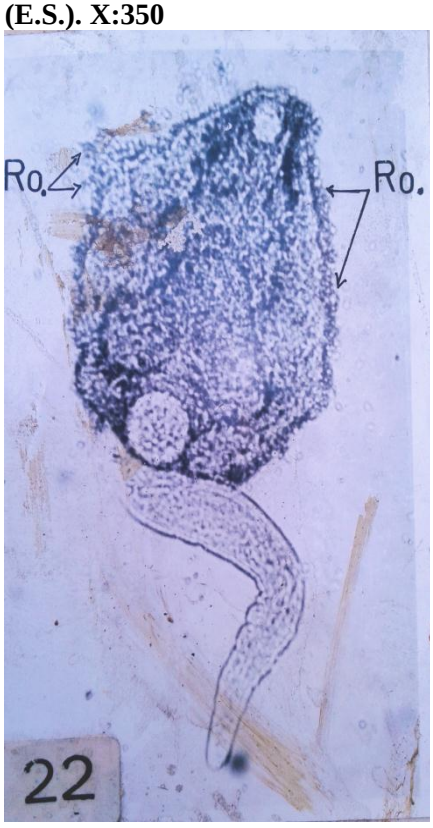

Fig (22): Photomicrograph of mature cercariae showing extrusion of cysto-genous rods (R.O) through the whole surface. X:120

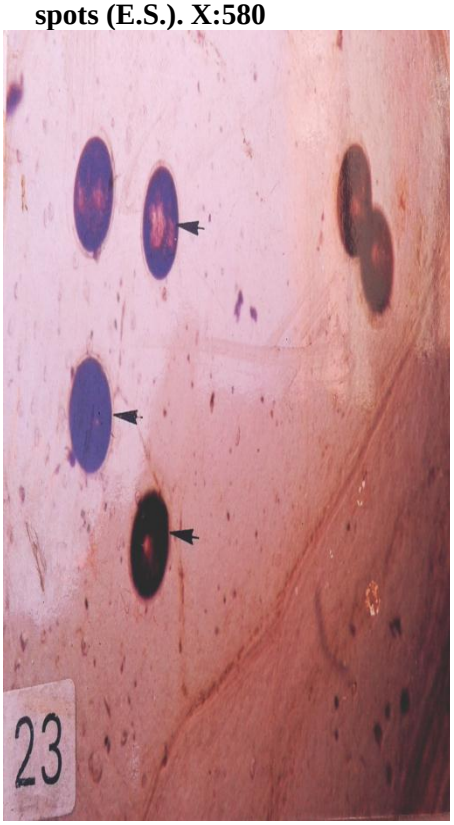

Fig (23): Photomicrograph of un-stained whole-mounted metacercariae on the lettuce leaves. X:45

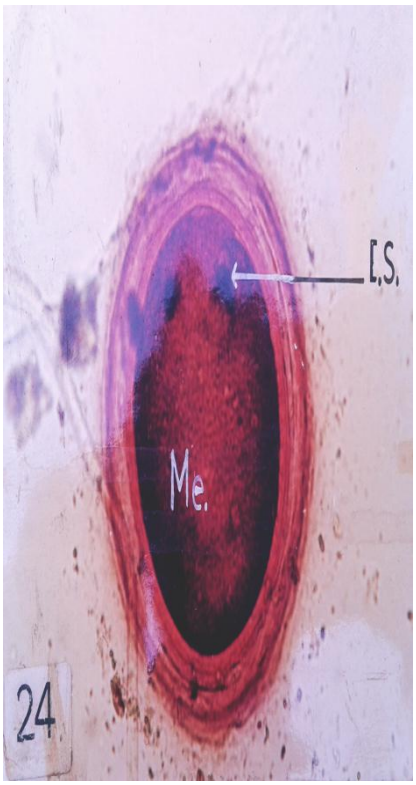

Fig (24): Photomicrograph of top view of metacercariae stained with aceta-carmine showing the body, and the eye spots. X:300

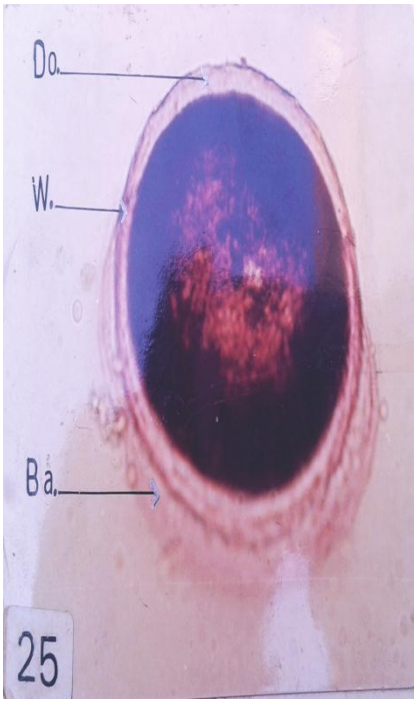

Fig (25): Photomicrograph of lateral view of metacercaria, stained with acetocarmine showing the dome shape (Do.), the base (Ba.) and the wall of the cyst (W). X:350

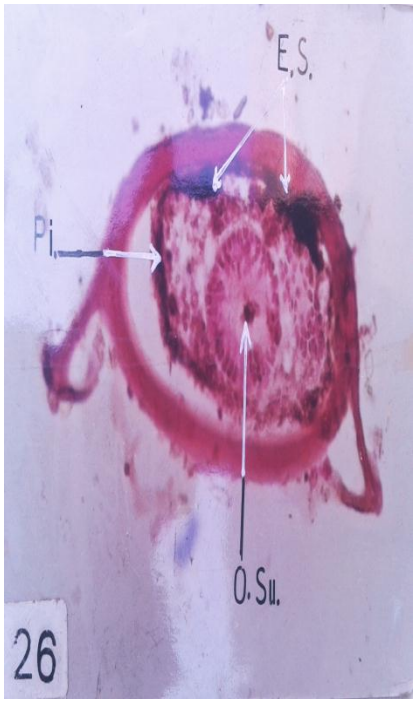

Fig (26): Photomicrograph of frozen section of metacercaria stained with acetocarmine showing the cyst wall (W), (E.S.), (O.Su.), (V.Su) and pigmented tegument (Pi). X:320

Fig. (27): Photomicrograph of Frozen section of the cyst stained with toluidine blue showing the various layers of the cyst including outer layer (O.L.), inner layer (I.L.) as well as pigmented wall (P.W.), the body, (V.Su.) and ventral blug (V.P.). X:300

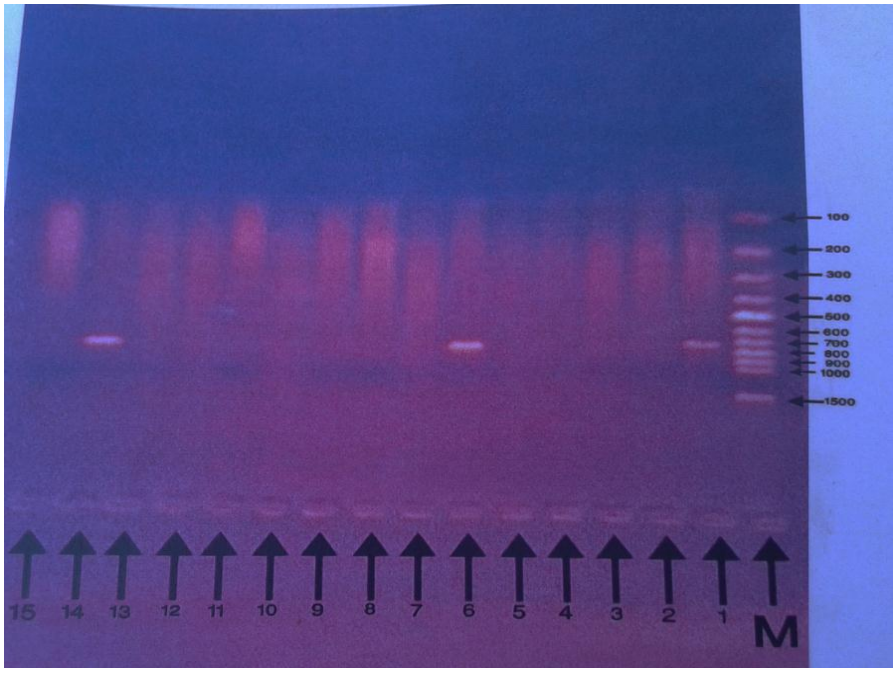

Fig. (28): Photomicrograph of detection of *Calicophoron microbothrium* (paramphistomatidae) DNA using polymerase chain reaction Mass–PCR. Lane m = molecular size ladder; lane 1 : Control, Lane 6 and 14 [positive samples by Mass–PCR at 796 bp.