

DECLARATION

**STIMULATION OF IMMUNE RESPONSE  
IN DOGS AGAINST ECHINOCOCCUS GRANULOSUS**

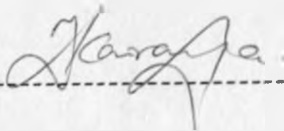
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Department of Public Health, Pharmacology and Toxicology,  
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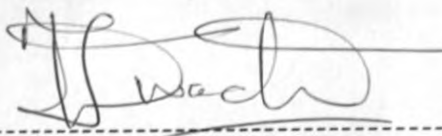
## DECLARATION

This project report is my original work and has not been presented  
for a degree in any other University.

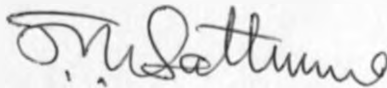


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This project report has been submitted for examination with our  
approval as the University Supervisors.



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## ABSTRACT

Hydatidosis is a cyclozoonosis that is caused by the larval stage of the cestode *Echinococcus granulosus*. It is of major veterinary and public health importance in many parts of the world. Several measures are available for its control but mass chemotherapy, using praziquantel is the main method used in dogs. Although the drug used is 100% effective against adult stages of this tapeworm it is not only expensive but also offers no protection against reinfection to treated dogs. Hence, today there exists a great need for the prolonged protection of dogs against this parasite.

In the present study vaccination of dogs against *Echinococcus granulosus* and their response to challenge infections were investigated. Intraperitoneal immunization of sixteen dogs was carried out using dead or live *Echinococcus granulosus* protoscoleces obtained from hydatid cysts of sheep origin, and later challenged with oral infection of about 34,000 protoscoleces in gelatin capsules. The degree of immunity stimulated was assessed by the rate of worm development and worm burdens in the vaccinated groups as compared to those in controls. Antibodies raised against these protoscoleces were detected using a crude protoscolex antigen in an enzyme linked immunosorbent assay.

Results showed that there were no significant differences in the levels of immunity developed by the vaccinated dogs as compared to the controls. Innate resistance was thought to have played a major role in the failure of establishment and development of *Echinococcus granulosus* worms in these dogs. It is then felt that this line of research utilizing crude protoscolex antigen offers little hope in inducing immunity in dogs against this parasite.

## CHAPTER ONE

### INTRODUCTION

*Echinococcus granulosus* is a small cestode of the genus *Echinococcus*, family Taenidae, whose larval stage is the aetiological agent of unilocular hydatidosis in both man and animals. The disease has a cosmopolitan distribution and it is of considerable medical, public health and economic importance. The importance arises from the infections it causes in both man and animals leading to lowered productivity, disabilities and sometimes death. Presently in humans no effective chemotherapeutic agent exists and such infections carry a poor prognosis. Surgery is the only practical treatment and it is occasionally done in combination with albendazole treatment for patients with active viable cysts (Nelson, 1990). However, this treatment may sometimes be followed by a high rate of recurrence with recurrent cases often presenting as multiple inoperable cysts which are invariably fatal (Macpherson, 1983).

Infections in livestock lead to the condemnation of offal resulting in high nutritional and monetary losses. These losses may be considerable in countries where this disease is endemic especially if these countries rely heavily on agriculture for their socio-economic development. In addition the cost of control programmes is also high (McConnell and Green, 1979).

Fortunately the disease can easily be prevented, except in its wildlife cycle, and the current control measures have attained high levels of success in New Zealand, Cyprus, Tasmania (Gemmell *et al.*, 1986a) and even attained eradication in Iceland (Dungal, 1946). Introduction of meat inspection services and education of the public to deny dogs raw offal would lead to a permanent control of the disease but, this has been difficult to achieve as it calls for a change

in human behaviour which may take many years to effect, especially in areas of low literacy levels. Praziquantel (Droncit®, Bayer, Leverkusen, Germany) is a cestocidal drug with a 100% efficacy against adult *Echinococcus granulosus* and it is widely used in hydatid control programmes for mass dog treatment every six weeks (Gemmell *et al.*, 1986a). However, the drug is not only expensive (Nelson, 1990), but it is also eliminated from the body within 24 hours (Andrews, 1978) and hence dogs can be reinfected soon after treatment. In addition, control by chemotherapy relies heavily on regular mass dog dosing and good public relations. Otherwise, control programmes may become unduly prolonged and expensive. The resources required by the present control measures may be difficult to meet in relatively poor nations and hence the need to develop a cheap and effective vaccine against canine echinococcosis to make the control of this disease feasible in such countries.

Various attempts to stimulate immune responses in dogs against adult *Echinococcus granulosus* have been made by several workers using different antigens obtained from different stages of this parasite (Clegg and Smith, 1978), while others have utilized antigens from heterologous cestodes (Rickard *et al.*, 1975). Various levels of success were achieved but these results lacked reproducibility mainly because workers used parasite material to challenge immunized and control dogs without testing protoscolece viability (Herd *et al.*, 1975). The present study was set up to investigate the level of immunity stimulated by intraperitoneal injection of either dead or live *Echinococcus granulosus* protoscoleces and the subsequent fate of these larvae in this location in the dogs.

## CHAPTER TWO

### LITERATURE REVIEW

#### 2. 1. The biology and life-cycle of *Echinococcus granulosus*

The hermaphroditic adult worm measures 3-9 mm in length and consists of a scolex and three or four segments, with the terminal one being gravid. It occurs in the small intestine of the domestic dog and wild carnivores where it sheds embryonated eggs in their faeces. These eggs are surrounded by a thick protective keratinized embryophore and can remain infective for long periods in the environment (Laws, 1968).

Intermediate hosts are primarily ungulates though primates, marsupials and rodents may be involved. These acquire the eggs from contaminated pastures or food, in the case of man. Once ingested digestive activity liberates the oncosphere which then gains access to a venule and finally lodges in body organs, mainly the liver or the lungs to develop to a hydatid larval stage. Production of protoscoleces occurs by asexual means from the germinal layer and over one million protoscoleces may be produced from a single cyst. This mode of reproduction may occasionally permit the formation of large numbers of genetically identical individuals from a single mutant offspring, leading to the development of new *Echinococcus granulosus* strains. However, the rate of cyst development and production of protoscoleces depends on an interaction between parasite factors and the species of intermediate host. Whereas the majority of cysts in sheep are large and highly fertile, those in most cattle and pigs are often degenerate and sterile (Thompson and Lymbery, 1988). In experimental infections in white mice rapid proliferation of large viable cysts occurs within the body cavities, sometimes killing the animals within three months (Heath, 1970).



Once the definitive hosts feed on fertile cysts, the protoscoleces are released in the stomach and rapidly evaginate under the influence of bile to attach to the small intestine. The attachment occurs by penetration of the scolex into the crypts of Lieberkuhn and occasionally into the lamina propria. Hence *Echinococcus granulosus* is regarded both as a luminal and a tissue parasite, an important consideration when studying immune responses in this host. The cestode then undergoes strobilisation to attain sexual maturity within six weeks though prepatent periods have been reported to vary in different *E. granulosus* strains. Strains of this parasite from Russia and Tasmania have been reported with prepatent periods of 5 weeks (Zhuravets, 1982; Kumaratilake *et al.*, 1983). The tapeworm may remain in dogs for at least two years during which it sheds a gravid proglottid containing about 500 eggs every two weeks (Gemmell, 1990). Domestic and wild dogs in Kenya and Australia respectively, have been known to harbour this cestode in very heavy burdens with no apparent ill health (Macpherson *et al.*, 1985; Jenkins and Morris, 1991). In rare cases the domestic dog may also act as an intermediate host for this parasite and other cestodes; *E. multilocularis* and *Taenia pisiformis* (Ivens *et al.*, 1969; Gracey, 1986; Geisel *et al.*, 1990)

## **2. 2. Global distribution of *Echinococcus granulosus***

*Echinococcus granulosus* has a cosmopolitan distribution occurring in all major climates, in a wide variety of hosts at various levels of prevalence (W.H.O., 1982). This pattern of distribution results from the existence of this parasite as a complex of strains which differ in their host range, infectivity to humans and involvement in sylvatic cycles (Thompson and Lymbery, 1988).

In the tundra region of the western hemisphere the strain that occurs, *E. granulosus canadensis*, is confined to a sylvatic cycle involving wolves and the large deers. It does not readily infect man or livestock (Cameron, 1960) and it is therefore of little public health importance. However, the sylvatic cycles in Australia which involve certain macropods and dingoes have been reported to readily infect domestic livestock and may act as continuous reservoirs of infections (Coman, 1972). The sheep strain of *Echinococcus granulosus* is the most widely distributed and responsible for endemic foci of this disease in Europe, Africa, South America and Australia where intensive sheep rearing occurs (Schwabe, 1969). This strain readily infects dogs and is responsible for most of human infections. Cattle are generally poor hosts for this parasite and the hydatid cysts present are often sterile and degenerated. Nevertheless, in South Africa and Switzerland the parasite causes fertile hydatid cysts in cattle, which are the most important intermediate hosts in those regions (Verster, 1965; Thompson *et al.*, 1984)). In Great Britain it is the dog-horse cycle that predominates and the parasite strain concerned has been reported to be of low infectivity to humans (Thompson and Smyth, 1975). The camel strain of the parasite occurs in parts of Africa and Middle East where fertile cysts are found in camels, often in contrast to other intermediate hosts in the same endemic area (Al-Yaman *et al.*, 1985).

In eastern Europe and the Soviet Union a pig strain of *Echinococcus granulosus* exists that is distinct from those occurring in other domestic animals and has low infectivity to humans (Pawlowski, 1985). While in India, a goat strain of this parasite reported to have a long prepatent period of 60-90 days occurs (Pandey, 1972).

### **2.2.1. Distribution of *Echinococcus granulosus* in Kenya**

In Kenya, infections in livestock are endemic in both the Turkana District and Maasailand while elsewhere they occur in low proportions. In these areas hydatid cysts are commonly seen in slaughtered cattle, sheep, goats and camels (Eugster, 1978; Macpherson, 1985). The sheep is the most important intermediate host, with hydatid cysts being readily infective to dogs, while those in cattle are often sterile. Macpherson (1981) reported a high prevalence of hydatid cysts (80%) in camels in Turkana District where it plays an important transmission role.

Human hydatidosis occurs in a hyperendemic proportion among the Turkana people of North-Western Kenya, who have the highest incidence rate yet reported (220 per 100,000 per year) (French *et al.*, 1982). The Turkana are pastoralists who own vast numbers of livestock together with numerous dogs which have been found to harbour very heavy *Echinococcus* worm burdens (Macpherson, 1985). These dogs not only share the same homesteads with their owners, but also play an important role as "nurse dogs" during child rearing stage (French *et al.*, 1982). These factors undoubtedly serve to enhance exposure of these people to hydatid infections. Furthermore the lack of a burial custom in this ethnic group gives dogs access to infected human corpses and so man in this region plays a unique role as an active intermediate host (Macpherson, 1983). In the rest of the country human hydatidosis is of very low occurrence, except in the Maasailand where many cases have been reported (Eugster, 1978).

Wildlife echinococcosis, whose transmission cycle mainly involves the lion and wild herbivores, has been reported to occur in the Maasailand and Serengeti regions of East Africa (Eugster, 1978), but has not been established in Turkana (Macpherson *et al.*, 1983).

The silver-backed jackals and Cape hunting dogs may also act as definitive hosts while fertile cysts have been found in wildebeest, warthogs and zebras (Nelson and Rausch, 1963). However, the relationship of this cycle to the domestic cycle operating in the same area is unclear (Macpherson *et al.*, 1983).

### **2. 3. *Echinococcus granulosus* infections in man**

The major concern about hydatidosis arises from the poor prognosis it carries in human infections, often resulting in many cases of severe disabilities and fatalities. Generally, human hydatidosis is a chronic disease manifested after a long prepatent period during which hydatid cysts grow large enough to impinge on local tissues, thus interfering with their normal function. The size and location hence clinical symptoms in patients, vary with the different strains of *E. granulosus* (Thompson and Lymbery, 1988). Cysts may occur in any of the body organs (Macpherson, 1983) but are mainly found in the liver and the lungs. Cysts have also been reported to stimulate immune reactions in the patients and lead to circulating immune-complexes in the plasma. These immune complexes may later be deposited in the renal glomeruli causing nephropathy (Vialtel *et al.*, 1981) and may also be responsible for lowered serum complement. Patients with the symptomatic disease are also at a risk of traumatic rupture of the cysts either spontaneously or at surgery. This may lead to the dissemination of protoscoleces, resulting in secondary hydatidosis with multiple inoperable cysts, or to a fatal anaphylactic shock in some patients. Fatal diffuse peritonitis may also follow the rupture of suppurative abdominal cysts (Kammerer and Schantz, 1984).

In Kenya it is speculated that the strains of *Echinococcus granulosus* which occur are particularly virulent in humans (French *et al.*, 1982). This may be supported by the observation that in Turkana District primary hydatid infections are characterized by large single cysts with a very rapid development rate. These cysts reach 5-10 cm in diameter within 3-5 years, both for the primary infections and for the recurrent cases (Macpherson, 1983). In contrast infections by *E. granulosus canadensis* which occurs in the western hemisphere, are asymptomatic in man (Cameron, 1960) and are mainly diagnosed immunologically.

## **2.4. Diagnosis of *Echinococcus granulosus* infections**

### **2.4.1 Parasitological diagnosis in man**

Diagnosis of human hydatidosis is based on clinical findings which are non-specific. Two groups of diagnostic aids are available and include those that detect hydatid cysts within the patients while the others detect circulating antibodies against *E. granulosus* (W.H.O., 1982). Cysts can be detected by ultrasonography, radiography, scintigraphy and computerized axial tomography. Of these, ultrasonography has proved to be the most convenient method in field diagnosis of hydatid cases especially in repeated mass surveys (Macpherson *et al.*, 1989). However, its application is limited only to diagnosis of intra-abdominal cysts.

*Echinococcus granulosus* infections in man stimulate readily detectable levels of specific antibodies that permit successful serodiagnosis by several methods (Williams, 1979). However, not all patients are positive in assays for specific antibodies and a combination of two or more of these methods measuring different classes of antibodies is recommended to increase specificity and

sensitivity (W.H.O., 1982). Nevertheless, the application of serodiagnosis is limited by cross-reactions between the antibodies raised against *E. granulosus* and other taeniid infections involving *Echinococcus multilocularis*, *Taenia saginata* and *Taenia solium*. Furthermore there has been a poor supply of parasite antigens and poor quality control for antigen preparations derived from different batches of hydatid cysts (Lightowers, 1990). Currently research is being carried out to identify antigen preparations specific for *E. granulosus* and to produce them by recombinant DNA technics to improve specificity and sensitivity as well as antigen supply for immunodiagnostic tests (Lightowers, 1990).

#### **2. 4. 2. Parasitological diagnosis in the domestic livestock**

Accurate diagnosis of hydatid infections in domestic livestock would be of great value in hydatid control programmes as it would permit the isolation and slaughter of infected individuals from the flock. However, at present this is only possible at slaughter during meat inspection. Serological diagnosis has so far been unsuccessful due to the production of very low or undetectable levels of specific antibodies in many animals with fertile cysts (Conder *et al.*, 1980) and the cross-reactivity between antibodies raised against other taeniids, mainly *T. saginata*, *T. ovis* and *T. hydatigena* (Yong *et al.*, 1978; Gathura, 1984). Furthermore, different strains of *E. granulosus* have been reported to give different antibody responses in different breeds of sheep, thus further complicating interpretation of serological results (Lightowers *et al.*, 1984).

Many immunodiagnostic tests have been tried mainly in sheep (Conder *et al.*, 1980; Craig *et al.*, 1981; Gathura 1984) but none has reliably differentiated between infections with *E. granulosus* and those of other commonly occurring cestodes. However, it has been

possible to differentiate between infected and none infected flocks of sheep (Lightowlers *et al.*, 1984). Hence at present there is little prospect for the development of a diagnostic test for use in livestock based on detection of antibodies in serum.

#### **2. 4. 3. Parasitological diagnosis in the definitive hosts.**

Accurate diagnosis of *E. granulosus* infections in dogs plays a major role in the effective implementation of hydatid control programmes. In the past this depended on purging dogs with arecoline hydrobromide, followed by examination of the purge-sample for this cestode. However, the test has low accuracy as some dogs fail to purge, while light infections are easily missed (Gemmell, 1968). In some regions the test has reported to underestimate the real prevalence rate by ten-fold (Wachira, 1990). Besides, the test poses the risk of hydatid infection to the control personnel, while the drug may cause strong side-effects in some dogs, sometimes with fatal consequences (W.H.O., 1982).

Autopsy in dogs, followed by the examination of their small intestines for this cestode, is the most accurate indicator of prevalence but it is limited in its application for surveillance purposes. However, it is still very useful in obtaining prevalence data on feral dog population and on wild carnivores.

Recently a species-specific indirect immuno-fluorescent test using an anti-*Echinococcus* oncosphere monoclonal antibody was developed that is capable of distinguishing eggs of *Echinococcus* from those of other morphologically identical taeniid species (Craig *et al.*, 1986). This test has been reported to have 100% specificity in detecting infected dogs and proves very useful in cases of mixed cestode infections in these dogs. An adjunct to this has been the use of perianal swabs or transparent tapes for the

identification of infected dogs by demonstrating *E. granulosus* eggs on their bodies. This test has also proved useful in Turkana District in assessing the degree of environmental contamination with such eggs (Craig *et al.*, 1988) and it may play a major role in future epidemiological studies, particularly in monitoring the progress of hydatid control programmes.

#### **2. 4. 4. Serological diagnosis of *E. granulosus* infections in dogs**

The successful development of an accurate serodiagnostic test for *E. granulosus* infections in dogs would be of great value in hydatid control programmes. Dogs infected with this cestode have been found to produce specific circulating antibodies against protoscolex antigen as early as 12 days post infection (Jenkins and Richard, 1986). These authors also noted that the antibody levels remained high throughout the infection period but fell rapidly to undetectable levels following purging and treatment with praziquantel and did not cross-react with antigens from other taeniid species. This then offered the possibility of early diagnosis of prepatent canine echinococcosis.

Gasser *et al.* (1988) have assessed the performance of such a serological test system based on ELISA for diagnosis of natural *E. granulosus* infections in dogs and obtained a sensitivity that proved superior to that obtained by arecoline hydrobromide purging. The test even offered the possibility of discriminating between prepatent and patent infections. It is anticipated that progress in this field will soon lead to the development of a diagnostic kit.



## **2. 5. Chemotherapy of hydatidosis in man.**

Surgery has been, for a long time, the only treatment available against human hydatidosis and involves the removal of the whole cyst together with the surrounding tissues (Rottcher, 1973). However, this is not always effective or possible in patients with multiple multiorgan hydatid involvement (Kammerer and Schantz, 1984). Complications following surgery also occasionally arise; these include fatal anaphylactic reactions following internal hydatid cyst fluid spillage and secondary hydatidosis arising from dissemination of protoscoleces. In Turkana region, the recurrence rate in patients following surgery has been reported to be as high as 14%, with many of these cases being inoperable and hence fatal (Macpherson, 1983). Besides, surgical treatment is always expensive and requires the provision of specialized staff and facilities.

Chemotherapy using the benzimidazoles derivatives, mainly mebendazole and albendazole, has been tried in treatment of human cases. Mebendazole, given orally at a recommended dose of 25-40 mg/kg, was found to have significant efficacy and causes the regression and sometimes complete destruction of the hydatid cysts in some patients (Kammerer and Schantz, 1984). However, it is only effective against single cysts, mainly of the liver and lungs. Patients with complicated multiple organ involvement respond variably while those with cysts in bone show little improvement.

Albendazole given orally at 10 mg/kg a day has been found to attain higher serum levels and to cure some of the infections refractory to mebendazole therapy (Morris *et al.*, 1983). In Kenya it has proved very effective in treatment of the rapidly growing Turkana cysts (Nelson, 1990) but it must be given daily for up to thirty days; and it is most effective in treatment of single cysts. Hence chemotherapy is not only expensive but also requires prolonged

follow-up of patients since the disease has been reported to relapse in previously treated patients that had been clinically stable for six years (Kammerer and Schantz, 1984). A combination of chemotherapy and surgery is today the preferred treatment for human hydatidosis.

### **2. 5. 1. Chemotherapy in the definitive hosts**

Many drugs have been tried in the control of canine echinococcosis but only praziquantel (Droncit<sup>®</sup>, Bayer, Leverkusen) has been found to be 100% effective against all strobilar stages of *Echinococcus granulosus* (Thakur *et al.*, 1978). Indeed mass treatment programmes have been successful in attaining a quick break in transmission in highly endemic areas (Gemmell and Johnstone, 1981). However, the drug has no ovicidal properties and it is eliminated from the body within 24 hours, (Andrews, 1978) meaning that such dogs can be reinfected soon after treatment. Consequently the six-weekly dosing regime adopted in control programmes has not only been expensive but a slight break in follow-up of cases would nullify earlier success. Hence there is great need for prolonged protection of dogs against this cestode if the control programmes are to succeed.

Currently the solution lies in the provision of a long-acting depot of praziquantel or in the development of a cheap vaccine against *Echinococcus granulosus* in dogs. Recent work done by Wachira (1988) has shown that it is possible to develop a system that would keep dogs free of such infections for periods of up to six months. This is achieved by use of a regulated-release praziquantel formulation given intraperitoneally.

## 2. 6. Control measures

There are two main hydatid control measures available; meat inspection followed by the denial of raw offal to dogs and the reduction of parasite biomass by mass dog treatment and/or reduction of dog population (Gemmell, 1979). The latter is achieved by killing of stray dogs or spaying of bitches that may be retained. However, the methods of application of these measures are dictated by the socio-economic factors prevailing in a country where such control is carried out (W.H.O., 1982); hence no control programmes have been identical. Nevertheless, in all cases a strong education programme adopted for the local community has assisted in gaining public support for the introduction and maintenance of control measures (Gemmell *et al.*, 1986b).

Control programmes have been initiated in more than fifteen countries (Wachira, 1988) and results from such programmes have shown that *Echinococcus granulosus* is not stable in its endemic cycle and easily responds to control (Gemmell, 1979). Rapid cessation of transmission to both man and domestic animals occurs at all age groups within a short time after the start of control measures. The major drawback of the present control measures lies in their heavy dependence on altering inherent patterns of human social behaviour such as the feeding of pets with cheaply available offal. For this reason some of these programmes become both protracted and uneconomical (Nelson, 1990).

In Kenya a pilot control programme was started in Turkana District in 1983 by the African Medical and Research Foundation team with emphasis placed on the education of the local people and reduction of the dog population (Macpherson *et al.*, 1986). Several problems have been encountered. These have mainly been attributed to the nomadic lifestyle of the Turkana people and the existence of a

high infection pressure to the dogs in this area, leading to a return of the infection rate in dogs to pre-control levels within 6 months (Wachira *et al.*, 1990). Hence there has been a need to maintain a six-weekly dosing programme of dogs with praziquantel in order to reduce the incidence of infection. This has been difficult and expensive to perform among these nomads. Therefore Wachira *et al.*, (1990) have recently suggested an alternative appropriate treatment schedule involving the dosing of dogs only during periods of high infection pressure.

### **2.6.1 Recent advances in control methods**

Recent advances in hydatid research have offered greater understanding of several aspects of this disease and the knowledge gained thereof is continually being incorporated into control programmes. Earlier programmes were based on arecoline purgation for both surveillance and education purposes but recent ones, such as in Chile, rely on a non-discriminatory dog-dosing programme with praziquantel every six weeks. These have reported a more rapid decline in prevalence of *E. granulosus* than the older programmes (Gemmell *et al.*, 1986a).

The recent development of an immuno-differentiation technic for distinguishing *Echinococcus* eggs from those of other taeniids has facilitated a more accurate diagnosis of infected dogs and provided a technic for routine assessment of environmental contamination with eggs (Craig *et al.*, 1988). The introduction of a portable ultrasonic scanner for field diagnosis of human hydatid cases (Macpherson *et al.*, 1989) has greatly improved on the speed and accuracy of collecting epidemiological data necessary for the initiation and monitoring of hydatid control programmes.

Studies in the biological strain variation of this parasite have led to the identification of strains whose prepatent periods differ

significantly from others (Thompson and Lymbery, 1988). Such knowledge is invaluable in adopting strategies to control this parasite especially in determining the interval for use in mass dog treatment programmes where worms must be expelled prior to patency.

Other control measures with great potential exist for hydatid control but are still in their prospective stages. Presently there is high optimism that a recombinant DNA vaccine against hydatidosis in livestock will be developed following the development of a similar vaccine against *Taenia ovis* in sheep (Lightowers, 1990). Vaccination in livestock would be preferable to chemical destruction of cysts, which would also inevitably lower the value of carcasses. Promising work also lies in the development of a long-acting depot of praziquantel to offer dogs prolonged protection against *Echinococcus granulosus* infections (Wachira, 1988).

The introduction of assays detecting circulating *E. granulosus* antigens will improve detection of infection in sera negative in other tests (Craig, 1986). Consequently early diagnosis of "silent" hydatid cases will permit early institution of treatment in a larger number of patients. Other desirable breakthroughs would be in the areas of serodiagnosis of hydatid infections in the intermediate hosts and the development of a vaccine in dogs against this cestode.

#### **2. 6. 2. Vaccinations in the intermediate hosts**

Natural and experimental *Echinococcus granulosus* infections in the intermediate hosts readily stimulate high protective immunity against challenge infections (Sweatman *et al.*, 1963). This is manifested as a reduction in the expected number of establishing larvae and/or an increase in the proportion of dead ones. However, this immunity does not seem capable of destroying hydatid cysts already established in the tissues and does not protect against

secondary hydatidosis (Gemmell *et al.*, 1986b). In all the vaccination experiments, viable *E. granulosus* eggs or activated oncospheres given intramuscularly have been the source of potent immunizing antigens. In these ectopic sites they undergo limited growth and hence stimulate strong immunity to reinfection.

Gemmell (1966) carried out such experiments in sheep and demonstrated high levels of protection (91.2%) against challenge infection and 99.6% protection against post-encystment survival of cysts. Heath *et al.*, (1981) vaccinated twenty-five sheep using activated *E. granulosus* oncospheres and it was only two sheep that developed only a single cyst each. Excretory/secretory antigens obtained from *in vitro* culture of *E. granulosus* oncospheres have been used to vaccinate sheep and have stimulated high degree of resistance to reinfections (Rickard and Williams, 1982; Osborn and Heath, 1982).

Despite this success, the unavailability of antigens has been a major drawback to the practical application of vaccination in the field. However, antigens from heterologous cestode species have been known to offer cross protection, though at lower levels. *Taenia ovis* antigens, for example, have been shown to cross-protect sheep against *E. granulosus* infections (Gemmell, 1966; Heath *et al.*, 1979). Recently there was a major break through in the making of the first highly successful recombinant vaccine for use in sheep against *Taenia ovis* (Johnson *et al.*, 1989). This vaccine has been reported to offer 98% protection to sheep and it is hoped that it will offer significant protection against *E. granulosus* in sheep and the other intermediate hosts (Lightowlers, 1990). This would greatly benefit hydatid control programmes since immunity to reinfection with the larvae has a central role in regulating natural transmission of the parasite (Roberts *et al.*, 1987).

### 2. 6. 3. Vaccination in the definitive hosts

The need for a vaccine against canine echinococcosis has long been recognized and many attempts at vaccination have been made by several workers (Turner *et al.*, 1955; Matov and Vasilev, 1955; Gemmell, 1962). These scientists used crude antigens obtained from hydatid material, hydatid cyst fluid, extracts of parasites and irradiated worms for vaccination. In some of the experiments partial immunity was stimulated in dogs and manifested as reduction in worm burdens, worm sizes, retarded sexual development and suppressed egg production in vaccinated individuals. In one case there was a total failure to take infection (Rickard *et al.*, 1977). Matov *et al.* (1955) reported that worms in the vaccinated dogs failed to reach sexual maturity even up to 88 days post-infection. However, in most of these experiments significant differences were not reported between immunized and control dogs. Moreover, results were often difficult to assess because workers frequently used different batches of protoscoleces to challenge immunized and control dogs without testing protoscolece viability (Herd, 1975). Movsesijan *et al.* (1968) carried out vaccination experiments using irradiated protoscoleces and achieved some success, but later Herd *et al.*, (1975) showed that some of the x-irradiated worms grew to produce infective eggs and being dangerous, research on this line was discouraged.

Later with the development of *in vitro* culture techniques for adult worms it became possible to collect excretory and secretory antigens from culture medium and use them for vaccination. Herd *et al* (1975) carried out the first such experiments and reported a highly significant suppression of egg production by worms in the vaccinated dogs, but no effect on the worm burdens. However, his later work (Herd, 1977) failed to

support these early experiments and it was speculated that there had been an interaction between the vaccine and the innate resistance of the host. Other attempts at vaccination using somatic antigens from oncospheres of a heterologous cestode, *Taenia hydatigena*, have also proved unsuccessful (Rickard *et al.*, 1975).

Although the adult *Echinococcus granulosus* worms occupy an intestinal site, and hence difficult to attack by immunological means, the parasite has been shown to penetrate through the mucosa into the lamina propria and behave as a tissue parasite (Smyth *et al.*, 1967). Undoubtedly the host is exposed to excretory and secretory antigens and these have been found to stimulate specific antibody responses within two weeks post-infection. The responses however disappear rapidly after purging (Jenkins and Rickard, 1985) and have not been proven to offer resistance to challenge infections. This may account for the observation that dogs can be re-infected soon after treatment.



## CHAPTER THREE

### MATERIALS AND METHODS

#### 3.1. Dogs

Twenty four two-month old puppies of unknown history and either sex were obtained from Kabete in Nairobi area. These were then randomly assigned to three experimental groups with eight members each. However, care was taken to ensure that littermates were distributed evenly in these groups. Members in the first and second groups were vaccinated with live and dead *Echinococcus granulosus* protoscoleces respectively, while those in the third acted as controls.

All the puppies were treated against helminth infections using pyrantel pamoate (Canex<sup>®</sup>, Pfizer, U.S.A.) at 50 mg/kg and praziquantel (Droncit<sup>®</sup>, Bayer, Germany) at 5 mg/kg and shampooed with Dudukrin<sup>®</sup> (Kapi, Nakuru, Kenya) to rid them of ectoparasites. Vaccinations were carried out against canine parvo-virus, distemper, infectious hepatitis and leptospirosis (Parvo-dog<sup>®</sup> and Caniffa<sup>®</sup>, Rhone Merieux, France). All the puppies were housed in dog kennels for 7.5 months in groups of threes and fed a commercial feed, Baysmix<sup>®</sup>, (Proctor and Allan, Nairobi), milk, boiled meat and water provided *ad libitum*.

#### 3. 2. Collection of hydatid cysts

Hydatid cysts were required for the preparation of live and dead-protoscolex vaccines, crude protoscolex antigen for use in ELISA and for oral challenge of *E. granulosus* infections in the dogs. The cysts for these purposes were obtained from livers and lungs of naturally infected sheep from Dagoretti and Kiserian slaughterhouses, near Nairobi. The protoscoleces were then aspirated from the fertile cysts

and washed in three changes of sterile phosphate buffered saline (PBS, 0.01M, pH 7.4). A sample of the protoscoleces was then transferred to a microscope slide and their viability determined by eosin dye exclusion test (Smyth and Barret, 1980). All samples examined and found viable, with viability of over 80%, were then pooled together.

### **3.3. Source of protoscolex vaccines**

The live-protoscolex vaccine was prepared directly from *E. granulosus* protoscoleces while the dead-protoscolex vaccine was prepared by first freezing the live protoscoleces at -70°C for 18 hours followed by thawing. Death of the protoscoleces was ascertained by microscopic examination for the absence of flame cell activity.

### **3.4. Immunization of dogs**

Prior to immunization the number of protoscoleces per ml was determined by repeated microscopic counts of protoscoleces in 0.02 ml aliquots. Using a 14 gauge needle, 0.1ml of well mixed protoscolex sediment (approximately 3,400) was injected intraperitoneally through the right paralumbar fossa of each experimental dog. This was followed by a three day 2 ml intramuscular injection of Combiotic<sup>®</sup> (400,000 units procaine penicillin and 0.5 gram dihydro-streptomycin) as an antibiotic cover. The dogs were then re-vaccinated 30 days later using the same procedure.

### **3.5. Experimental infection of dogs with *E. granulosus***

To each of the dogs 0.20 ml of packed *E. granulosus* protoscoleces (about 180,000) in a gelatin capsule were administered *per os* by opening the dog's mouth and placing the capsule at the back of the tongue then allowing the dog access to some milk.

### **3.6. Autopsy**

This was done 32 days post infection. The dogs were fasted for 24 hours and then killed by an overdose of hyperconcentrated magnesium chloride solution injected via the cephalic vein. Immediately the abdominal and thoracic cavities were opened up and an examination for hydatid cysts was carried out. The small intestine was then loosened from the mesentery and cut up at the anterior part of the duodenum and at the junction with the large intestine. The contents were gently milked out and examined for worms. The small intestine was then slit longitudinally using gut-scissors. This was followed by washing it in a beaker containing saline at 37°C and leaving it to stand for 15 minutes to permit the detachment of worms from the mucosa. It was then transferred into another beaker of tap water for 30 minutes and a final washing done, making the total volume of washings to 1750 ml. The mixture was then thoroughly stirred and 1 ml transferred to a petri-dish using a Pasteur pipette and a worm count done under a dissecting microscope. This was repeated ten times and the total number of worms in 10 ml determined. The figure obtained was then multiplied by the dilution factor of 175 to approximate the total worm burden in each dog. All the worms counted were preserved in 40% formaldehyde-saline solution.

#### **3.6.1. Preparation of worms for morphological examination**

Worms preserved in 40% formaldehyde-saline were transferred to a petri-dish and washed in several changes of distilled water for 30 minutes and then stained with aceto-alum carmine. Dehydration was carried out using increasing concentration of industrial methylated spirit. The worms were then cleared in clove oil and mounted in Permount<sup>®</sup> and examined under the microscope.

### **3.6.2. Microscopic examination of the worms.**

Detailed microscopic study of the worms was carried out to assess the level of somatic and germinal development by the examination of the number of proglottids, genital pore, and the presence of testes, ovaries the uterus and eggs.

### **3.6.3. Data analysis.**

Comparison of the antibody responses in the experimental dogs was done by the use of student t-test while that of worm burdens was done using a one-way analysis of variance method, both tests at 95% confidence level.

### **3.7.1. Blood sampling.**

Three blood samples were obtained from each dog; before immunization, two weeks after booster vaccination and two weeks following challenge infection. 10 ml of blood was drawn aseptically from the jugular vein into a sterile universal bottle. It was left overnight at room temperature and then centrifuged at 10,000 g for ten minutes. To 5 ml of the serum obtained sodium azide was added at a rate of 0.001% to inhibit microbial growth. The serum was then frozen at -20°C in polystyrene tubes till required.

### **3.7.2. Preparation of crude protoscolex antigen for use in ELISA.**

Viable *E. granulosus* protoscoleces obtained as described earlier were disrupted by sonication in ice for 15 minutes. The sonicate was centrifuged at 10,000g at 4°C for 15 minutes.

### **3.7.3. Enzyme Linked Immunosorbent Assay.**

Flat bottomed Microtiter® plates (96 wells) (Dynatech Laboratories, Virginia, U.S.A.) were coated with 100 µl per well of the protoscolex antigen diluted in 0.06 M bicarbonate-carbonate buffer, pH 9.6. The plates were then incubated in a humid chamber for 12-14 hours at room temperature (approx. 23°C). These antigen-sensitized plates were washed 3 times with 300 µl per well of phosphate buffered saline containing Tween 20, leaving it for a few minutes and tapped dry. Three dilutions 1/5, 1/10 and 1/20 of test serum were prepared using the serum diluent (PBS pH 7.5 and Tween 80) and 100 µl was transferred to each well of a triplicate. Reference negative, no antigen- and no serum- controls were included in each plate. The negative control serum was obtained from a pooled sample of sera drawn from all the puppies prior to immunization. The plates were incubated at 37°C for one hour, emptied, washed as above. Fifty microlitre of horse-radish peroxidase-conjugate goat anti-dog IgG diluted 1/500 in conjugate diluent was then added and incubated for one hour at 37°C. The plates were then emptied, washed as above and tapped dry. A hundred microlitre of the O-phenylenediamine (OPD) added to each well and developed by agitation for 4 minutes then incubated at 37°C for 30 minutes and the reaction stopped with 2% sulphuric acid. An ELISA plate reading spectrophotometer was used to determine the absorbance at a wavelength of 490nm and was blanked against the substrate. Mean spectrophotometric reading of individual test serum were then calculated from the triplicate readings.

## CHAPTER FOUR

### RESULTS

Prior to immunization all the sera tested proved negative for anti-*Echinococcus granulosus* protoscolex antibodies in all the dogs. Antibody responses were detected 2 weeks following booster vaccination with either live or dead vaccines. These responses were readily detected on ELISA but the two vaccine preparation elicited similar levels of responses in the various groups of dogs (figure 1). The mean optical density readings among the live-vaccine group was  $0.325 \pm 0.056$  while that in the dead-vaccine group was  $0.314 \pm 0.051$  respectively, but were not significantly different ( $p > 0.05$ ).

After challenge infection the antibody response in the two vaccinated groups were slightly amplified (figure 1) to give in the live-vaccinated a group mean of  $0.551 \pm 0.1053$  while that of the dead-vaccine group was  $0.554 \pm 0.1365$  but these were also not significantly different ( $p > 0.05$ ). The challenge infection in the control group also elicited some level of immune response in 7 of the 8 control dogs.

At autopsy 10 of the 24 dogs, (seven immunized and three controls) were found to harbour no worms. The worm counts differed between the three groups of dogs and among the individual group members as shown in table 2. The highest worm counts were found in the control dogs 19 (25,600) and dog 21 (15,575) and this resulted in the total worm count in the control group being about twice that in either of the immunized groups.

Microscopic worm examination showed that the development rates in these worms also differed between the groups of dogs and in the individual dogs, but the overall level of development was similar as evidenced in table 1. Majority of the worms examined had only reached the second proglottid stage of development and were at the

very early stage of sexual maturity marked by the presence of testes and genital pore in the second proglottid. The most advanced growth was found in those worms with the third proglottid just budding off which were obtained from dogs 1 and 2 (live-vaccinated), 13 (dead-vaccinated) and 19, 21 (controls). In none of the worms examined was there found a dilated uterus or the presence of eggs. However, in dogs 3 (live-vaccinated) and 23 (control) a large proportion of the worms recovered had only developed to the first proglottid stage.

At autopsy small white fibrotic nodules (about 2 mm in diameter) were found on the mesentery, liver and spleen capsules of all dogs vaccinated with live *E. granulosus* protoscoleces but not in the other dogs.

Table 1 : number of proglottids for worms from control and immunized dogs.

Number of proglottids	% worms		
	Live-vaccinated (110)*	Dead-vaccinated (134)*	Control (249)*
0	0	1	0
1	14	10.5	12
2	80	78.9	87
3	6	7.9	5.4
4	0	0	0

\* number of worms examined.

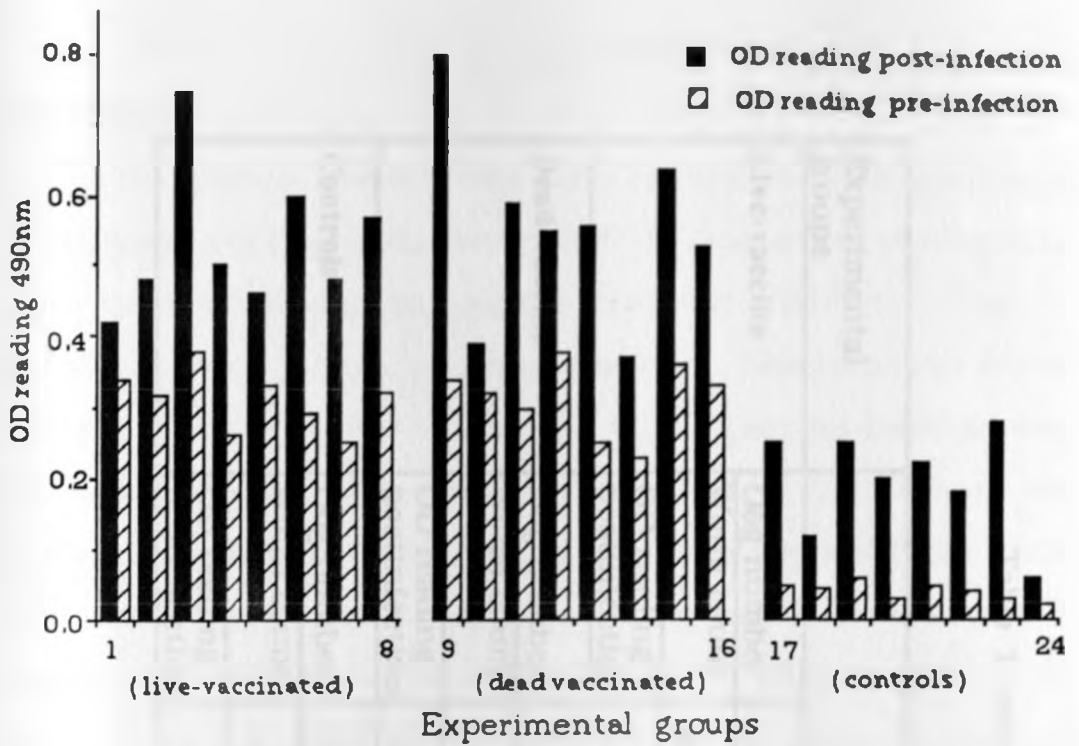


Figure 1; antibody levels before and after challenge infections.

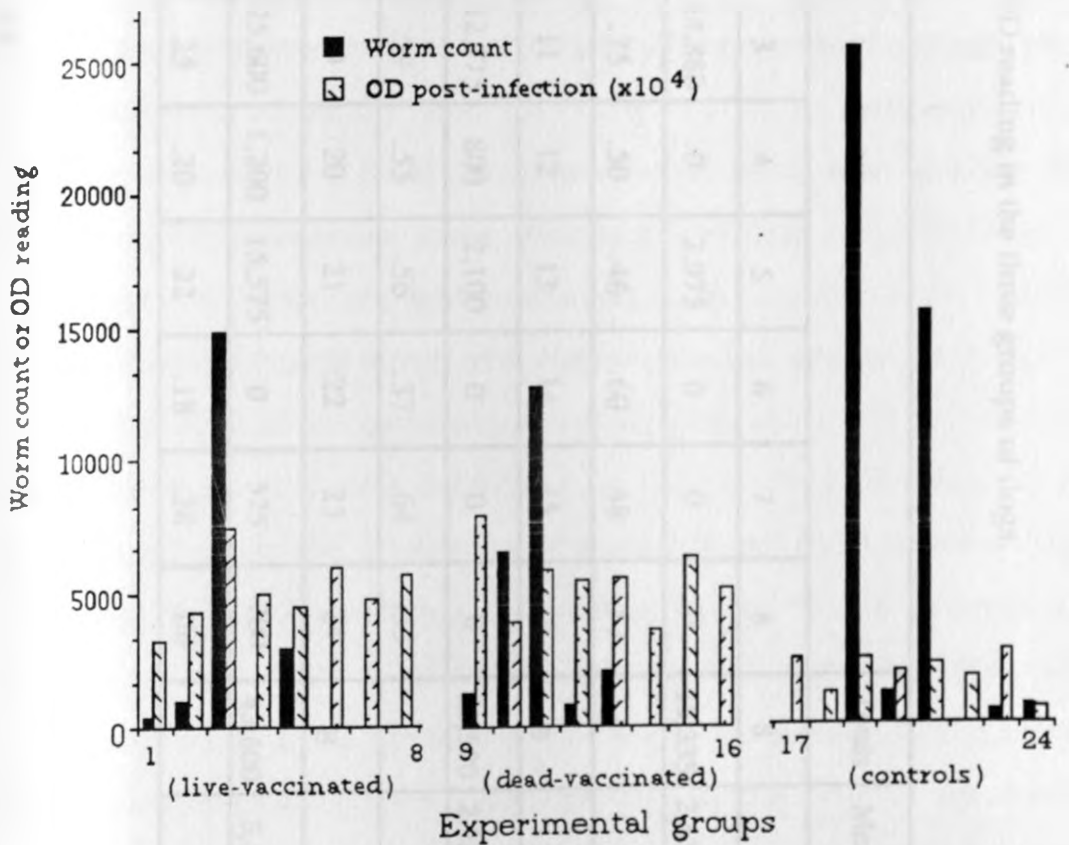


Figure 2 ; worm count versus OD reading in the three groups of dogs.



**Table 1;** worm count and OD reading in the three groups of dogs.

<b>Experimental groups</b>										<b>Totals</b>	<b>Means</b>
<b>Live-vaccine</b>	<b>Dog number</b>	1	2	3	4	5	6	7	8	8	
	<b>Worm count</b>	480	1,000	14,880	0	2,975	0	0	0	<b>19,335</b>	<b>2,416.9</b>
	<b>OD reading postinfection</b>	.42	.48	.75	.50	.46	.60	.48	.57		
<b>Dead-vaccine</b>	<b>Dog number</b>	9	10	11	12	13	14	15	16	8	
	<b>Worm count</b>	1,225	6,600	12,775	800	2,100	0	0	0	<b>23,500</b>	<b>2,937.5</b>
	<b>OD reading postinfection</b>	.80	.39	.59	.55	.56	.37	.64	.53		
<b>Controls</b>	<b>Dog number</b>	17	18	19	20	21	22	23	24	8	
	<b>Worm count</b>	0	0	25,600	1,200	15,575	0	525	700	<b>43,600</b>	<b>5,430</b>
	<b>OD reading postinfection</b>	.25	.12	.25	.20	.22	.18	.28	.06		

## CHAPTER FIVE

### DISCUSSION

The present experiments have shown that intraperitoneal immunization of dogs with live or dead *E. granulosus* protoscoleces leads to stimulation of an antibody response readily detectable on ELISA employing a crude protoscolex antigen. This response, noticed 2 weeks following booster vaccination, was indistinguishable between the dead or live antigens. Following oral challenge of dogs with protoscoleces the antibody response was slightly amplified in the vaccinated dogs. However, this immune response seemed to offer no significant protection against establishment of this parasite in the dogs since the antibody levels could not be correlated to the worm counts (figure 2), neither to the suppression of worm development (table 1).

Past immunization experiments (Gemmell, 1962; Herd, 1977) which have reported partial protection of the vaccinated dogs were also unable to relate the immunity observed to the antibody levels. Even in the most successful experiments of Herd *et al.* (1975), the strong antibody response found in immunized dogs was unrelated to the degree of immunity and moreover, this response was entirely absent in control dogs which also showed similar level of resistance.

Though it is common for different members of a host population to respond differently to helminth infections, it is surprising that in the present study complete failure of infection was observed in 10 out of 24 dogs while seven of the other dogs developed high worm burdens (> 2000 worms), though fed on the same batches of viable protoscoleces. Failure of infection in dogs from this cestode has in the past been reported to arise due to age, breed and even innate resistance. Mature dogs have been found to be more resistant to *E. granulosus* infections than young ones, while innate

resistance has been reported to occur at a significant level in the Beagle breed of dogs (Gemmell, 1962; Lubke, 1973; Herd, 1977). In the present study, age factor was standardized by use of age-matched groups, but the breed factor was hard to assess as mongrel puppies were used. It was then speculated that innate resistance played a major role in the failure to establish infections in these dogs. This speculation was strengthened by the observation that three littermates (dog no. 6, 14 and 22) failed to take up the infection despite each having undergone different treatment (table 2).

Exposure of *E. granulosus* protoscoleces to dog's bile lead to rapid evagination followed by attachment to the small intestinal wall. In different breeds of dogs bile has been reported to differ in its composition (Smyth and Haslewood, 1963) and though this may slow evagination of protoscoleces, it is unlikely to play a major role in mediation of this resistance since evagination has previously been shown to occur even in saline (Smyth and MacManus, 1989). It is therefore likely that the evaginated scoleces were expelled following attachment to the gut wall and this probably accounts for the antibody responses observed in the control dogs, even those harbouring no worms (dog no. 17, 18 and 22).

In the small intestine *E. granulosus* has been found to be intimately associated with the lamina propria, and therefore exposed to easy attack by the host's effector systems. Complement is likely then to have been involved in destruction of these newly evaginated scoleces since the cestode tegument has been shown to be highly susceptible to complement attack (Herd, 1976). Following *in vitro* incubation of *E. granulosus* worms and protoscoleces in normal or immune dog's sera, Herd demonstrated that lysis occurred readily within 30 minutes. Hence the possibility that the dogs showing resistance may have had a genetic factor responsible for a high level

production of complement. It is also probable that these dogs may in addition, have had a higher level of non-specific cellular immunity that provided a hostile environment to these scoleces.

The rate of somatic and germinal worm development was found to vary in worms from dogs having been similarly vaccinated. This had been previously reported by other workers (Gemmell, 1962; Ford, 1977). This difference could not be attributed to immunization since the overall growth rate was similar in the three groups of dogs. An important observation however, was that even in the heavily parasitized control dogs, this rate of worm development was markedly slower in this group of Nairobi dogs when compared to that reported earlier in the Turkana dogs fed similar hydatid cysts Machira (1988). Besides, this degree of development at 32 days post infection was only comparable to that at 26th day development using the criteria established by Smyth *et al.*, (1967) for the optimal development of *E. granulosus* in the definitive host. This generally depressed growth rate could most likely be attributed to host factors in this group of Nairobi dogs, since infection is a function of the host-parasite relationship. Furthermore, certain breeds of dogs have been reported to naturally permit easy establishment of heavy *E. granulosus* worm burdens (Macpherson, 1985; Jenkins and Morris, 1991).

Live protoscoleces injected intraperitoneally in dogs failed to develop to hydatid cysts. Their development seems to have been arrested quite early, leading to formation of small fibrotic nodules in mesentery, liver and spleen capsules. Though no development of protoscoleces had been anticipated from any cysts that would develop, small viable cysts were expected. This is supported by the previous reports that the domestic dog can harbour larval stages of *T. uniformis*, *E. multilocularis* (Ivens *et al.*, 1969; Geisel *et al.*, 1990)

and even *E. granulosus* (Gracey, 1986). However, the location of these nodules does suggest that the survival, migration and early growth of the protoscoleces in these sites had occurred but later limited by encapsulation. In contrast, experimental induction of secondary hydatidosis in the intermediate hosts is often very successful and factors responsible for this discrepancy in such infections are still unclear today. It is for this reason that attempts at induction of hydatidosis should be carried out in immunosuppressed dogs which would probably permit larvae development. These cysts would serve to offer a prolonged exposure of the host with parasite antigens and probably stimulate some immunity to challenge infections.

Detection of antibodies against *E. granulosus* infections by ELISA using a crude protoscolex antigen was successful in this study and confirms similar work done by Gasser *et al.*, (1988). By selecting the discrimination absorbance value to be 0.2 the test would detect 5 out of 8 infected control dogs. Similar application in detecting infection in the immunized groups was not possible. This would be complicated by the antibody response already existing prior to infection and it would be difficult to ascertain that these dogs would have tested positive were it not for vaccination. Moreover, since crude protoscoleces were used for vaccination, antibody responses were probably stimulated against several protoscolex antigens. Hence the amplified response observed in these dogs may not necessarily have been due to infection, but rather resulted from hyperimmunization with protoscolex antigens following oral challenge irrespective of any establishment of infection.

From these experiments, it can be concluded that although innate resistance with no observable immunological basis may have played an important role in determining the worm counts obtained, vaccination with *E. granulosus* protoscoleces offers dogs no

significant protection to homologous challenge infection. This may be due to scarcity of immunogenic antigens against adult *E. granulosus* in protoscoleces or to poor presentation of these antigens via the intraperitoneal route. Gemmell (1962) had earlier pointed that vaccinating dogs with adult tapeworm material gave better protection than with protoscolex antigens.

The intraperitoneal route has repeatedly been proved very useful in induction of high and even absolute immunity resistance to larval cestodes; *Taenia taeniaeformis* and *Taenia ovis* in rats and lambs respectively (Miller, 1932; Rickard and Bel, 1971). However, Rickard *et al.*, (1975) failed to immunise dogs against this parasite using the same route. It seems then, intraperitoneal vaccination against this cestode is not useful in the definitive host. Indeed the only successful vaccination of dogs against this cestode were achieved by Herd *et al.* (1975) using secretory antigens obtained from *in vitro* culture of adult stages and given intramuscularly.

Protoscoleces been poor antigen source for vaccination against *Echinococcus granulosus* in the dogs has been reported (Herd *et al.*, 1975). It is then plausible that, the suggestion by Ito and Smyth (1987) that successful immunization against adult *Echinococcus granulosus* stages is best accomplished by use of antigens derived from adult stages is true.

Presently the major hindrance to successful vaccination of dogs against canine echinococcosis lies in the identification of functional antigens to permit vaccination. There also lies a great need for genetic characterization of innate and acquired resistance in dogs to permit the proper interpretation of results obtained from such trials.

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