IMMUNODIAGNOSIS OF BOVINE CYSTICERCOSIS USING SELECTED ANTIGENS

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other university.

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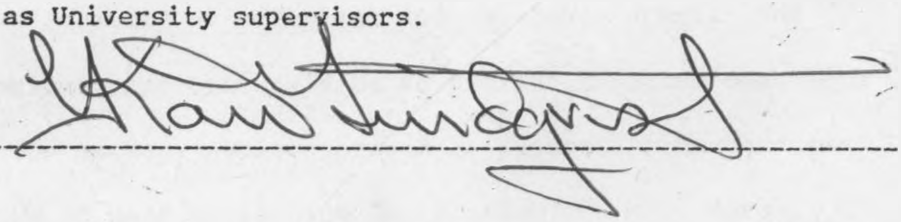
This thesis has been submitted for examination with our approval as University supervisors.



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SUMMARY

Despite significant advances in the development of cestocidal agents and improved understanding of the epidemiological factors affecting the transmission of tapeworms, there has been in fact an increase in human taeniasis and bovine cysticercosis due to Taenia saginata during the last two decades.

The control measures which have been tried have largely failed to achieve the expected results. In theory, this cyclozoonosis can easily be controlled. In order to break the cycle of man-cattle-man transmission, two approaches, or a combination of the two, are evident. Firstly, the eradication of human taeniasis through appropriate treatment, thus eliminating the source of infection for cattle. This approach has largely failed because of the logistics involved, in spite of the availability of efficient and non-toxic drugs. The second logical approach would be to prevent infected meat from reaching the market. This approach is presently in operation in the form of meat inspection legislation and procedures, which inter alia are aimed at the detection of T saginata metacestodes to prevent marketing of infective beef. Unfortunately, experience and careful studies have shown that the meat inspection procedures are unable to detect light infection, resulting in the marketing of considerable quantities of infective beef.

In view of the present unsatisfactory results of attempts to control human taeniasis and bovine cysticercosis, it could be surmised that a serological diagnostic method might be of value both in the ante-mortem as well as post-mortem diagnosis of bovine cysticercosis. It is envisaged that a reliable ante-mortem diagnostic method would provide the livestock farmer with means of identifying infected animals. Such animals may be disposed of immediately to prevent the losses incurred in feeding of animals which may be condemned or downgraded at the time of slaughter. A serological test may also assist in identifying infected animals at the time of slaughter, despite of negative findings during meat inspection. In the future, cattle herds could be screened using a serological method. Those giving positive reactions may be considered for treatment when an efficient drug becomes available. So far, however, drug treatment of bovine cysticercosis has not been attempted except on an experimental basis. Finally, it can be envisaged that the present meat inspection procedure can be simplified through the introduction of a serological method for bovine cysticercosis, in a way similar to what has been done for porcine trichinellosis.

The fundamental aim of the present study was to examine the antigenic components of T.saginata metacestodes in an attempt to select antigen(s) suitable for the use in serological tests. This required investigations of the complexity of antigenic determinants shared with other common

parasites of cattle, the selection of antigen(s) possessing adequate specificity and the establishment of sensitive serological methods using these antigens.

In addition to the potential practical implications of the development of such immunoassays, these investigations might contribute to the understanding of fundamental aspects of the immune responses to parasitic infections in general.

Chapter I describes the establishment of a reference system for the antigens of T saginata metacestodes. Fifteen antigens were defined by the Laurell crossed immunoelectrophoresis technique. Antigens were given abitrary numbers starting with the most anodic components. Antigens 1,3,4,5,7,8,9,10,11, and 13 were found to be shared between T. saginata cysts and the adult worm, while Antigens 1,2,4,6,7,9,10,12 and 13 were common among cestodes. Antigens 10,14 and 15 were shared with commonly occurring nematodes and trematodes of domestic animals.

Antigens 4, 8 and 11 were considered potentially useful and were selected for further study. Antigen 4 was found to be common among all cestodes examined, while Antigens 8 and 11 had a more restricted distribution. Antigen 8 was only shared with T. saginata and its metacestode. Specific antisera to these antigens were prepared in rabbits using immunoprecipitates obtained in immunodiffusion tests using rabbit antiserum to T saginata metacestodes, and metacestode extract as antigen.

Chapter II describes the isolation and purification procedures of these antigens. All the three antigens were isolated using an affinity chromatography technique. The antigens were analysed by isoelectric focusing and polyacrylamide gel electrophoresis in which sodium dodecyl sulphate was incorporated (SDS-PAGE). Antigens 4,8 and 11 were found to have molecular weights of 63 kd, 260 kd, and 68 kd and gave pI values of 5.20, 6.95 and 5.50, respectively.

Antigen 8 was found to consist of three components of molecular weights 110 kd, 72 kd and 61 kd. All these components showed reactions of identity in immunodiffusion tests, indicating that they shared the same epitopes.

Chapter III describes an attempt to determine whether the three antigens could be used for the serodiagnosis of bovine cysticercosis. The "sandwich" enzymeimmunoassay and a radioimmunoassay were first used to evaluate these antigens using sera from experimentally infected animals. Since antibodies to all three antigens could be detected in experimentally infected animals, the applicability of these antigens in detecting antibodies in naturally infected animals was evaluated.

The "sandwich" enzyme immunoassay using Antigen 8 gave a sensitivity of 66%, a specificity of 40% and a predictive value of 56%. Antigen 11 gave a sensitivity of 55%, a specificity of 48%, and a predictive value of 52%. Antigen 4 gave a sensitivity of 45%, a specificity of 45% and a predictive value of 46%.

There was no statistically significant difference between the sensitivities obtained with Antigen 8 and 11 in the enzyme immunoassay ($t = 1.925$; $P > 0.05$). There was a significant difference in the sensitivities obtained when Antigen 8 was compared with Antigen 4 ($t = 2.528$; $P < 0.05$) Antigen 8 was more sensitive than Antigen 4. A comparison between Antigen 11 and Antigen 4 also gave a significant difference in sensitivity ($t = 5.885$; $P < 0.05$) with Antigen 11 giving a higher sensitivity than Antigen 4. There was no statistical difference between the specificities obtained when Antigen 8 was compared with Antigen 11 in enzymeimmunoassay ($t = 0.980$; $P > 0.05$), while there was a statistically significant difference in specificities shown by Antigen 8 and Antigen 4 ($t = 3.261$; $P < 0.05$), with Antigen 8 giving a higher specificity than Antigen 4. Antigen 11 gave a higher specificity than Antigen 4 ($t = 2.297$; $P < 0.05$).

In the radioimmunoassay, using labelled antigens, a sensitivity of 82%, a specificicity of 25% and a predictive value of 58% were obtained with Antigen 8. A sensitivity value of 76%, a specificity of 30% and a predictive value of 58% were obtained with Antigen 11. Antigen 4 was not evaluated in this assay.

There was no statistical difference when the sensitivities obtained with Antigen 8 were compared with Antigen 11 ($t = 0.712$; $P > 0.05$). Neither was there any difference in the specificities obtained with these antigens in radioimmunoassays ($t = 1.054$; $P > 0.05$).

There was a statistical difference in sensitivity when Antigen 8 was used in radioimmunoassay, as compared to its use in enzymeimmunoassay ($t = 3.803$; $P<0.05$) with radioimmunoassays using Antigen 8 giving a higher sensitivity. There was also a difference in specificities obtained in radioimmunoassay as compared to enzymeimmunoassay ($t = 2.042$; $P< 0.05$) with enzymeimmunoassay using Antigen 8 giving a higher specificity. A significant difference in sensitivity was obtained when Antigen 11 was used in a radioimmunoassay as compared to enzymeimmunoassay ($t = 2.677$; $P< 0.05$) with enzyme immunoassay using Antigen 11 being more specific.

This difference in sensitivities and specificities could be attributed to the use in the radioimmunoassay of labelled antigens, while in the "sandwich" enzyme immunoassay an anti-bovine IgG conjugate was used.

The high percentage of apparent false positive reactions obtained with the immunoassays may in fact be true positive reactions because of the poor reliability of meat inspection procedures which were used as the definitive parameter for the diagnosis. On the other hand, false positive reactions may have been due to abortive infections as the results of the ingestion of senescent eggs. Such eggs may fail to develop into mature cysts but nevertheless trigger an immune response and give rise to detectable antibody levels.

There are several ways in which the immunoassays described in this study may be applied. For example, the

enzymeimmunoassay using Antigen 8 and 11 may be used for the selection of animals which are heavily infected. Such animals could be separated for treatment or slaughtered at an early age, thereby saving the farmer from keeping animals which can be downgraded or condemned at the time of slaughter. The same method could be used in the selection of animals which are to be taken to a feedlot for fattening.

These assays could also be used for the detection of lightly infected animals as an adjunct to the current meat inspection procedure. This could justify the cold storage of all carcasses from animals which gave a positive reaction in the immunoassay. On the other hand, animals which gave negative reactions in the serological tests could be subjected to a less vigorous inspection.

The presence of antibodies to T. saginata metacestodes in cattle from a certain farm or area may also be used as indirect way of identifying persons with taeniasis. The treatment of persons identified in this way should be an intergral part of control measures for bovine cysticercosis.

In this study it has been shown that the selection of certain antigens of T. saginata metacestodes can easily be made from complex mixture of antigens by the use of a reference pattern based on the Laurell crossed immunoelectrophoresis technique. The same reference system can be used to elucidate the relationship of these antigens with those of other parasites.

The selection of three antigens which were consistently present in T. saginata metacestodes and absent in most other prasites was considered the most appropriate choice of antigens for use in specific serodiagnostic tests for bovine cysticercosis. Encouraging results were obtained with Antigens 8 and 11 and these antigens could be used as an adjunct to current meat inspection procedures.

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Dedicated to:

All my teachers,

My parents,

My family and

All those who made this study possible.

"No animal has been responsible for more hypotheses, discussions and errors than the tapeworm"

C. Davaine, 1860.

IMMUNODIAGNOSIS OF BOVINE CYSTICERCOSIS USING SELECTED ANTIGENS

1. GENERAL INTRODUCTION

Parasitic infections of animals slaughtered for human consumption represent well recognized public health hazards. There are many infections which man can acquire through the consumption of raw or undercooked meat. These include infections such as toxoplasmosis, sarcosporidiosis as well as helminth infections like trichinosis and taeniasis. So far, no practical methods have been developed which can reliably demonstrate these infectious agents in animals at slaughter.

The situation is considerably different for zoonotic helminth infections where extensive knowledge of life cycles, modes of transmission and theoretically sound preventive measures have resulted in specific steps in an attempt to control these infections. For example, the introduction of conventional meat inspection procedures has undoubtedly contributed considerably to reducing the dissemination of meat heavily infected with Taenia saginata metacestodes (Pawlowski and Schultz, 1972). Nevertheless, it is evident that routine meat inspection, no matter how efficiently performed, cannot completely achieve elimination of human taeniasis and bovine cysticercosis (Geerts et al., 1977). In fact, the low reliability of standard meat inspection is reflected in the steady increase of taeniasis on a world wide scale (Pawlowski, 1971; Olteanu, 1982; Mayr, 1984). For instance, the reported incidence of bovine cysticercosis in the industrialized countries is 0.5-2% based on records obtained

through routine meat inspection procedures, while the real prevalence as revealed by intensive carcass examination has been shown to reach even 10% in France, Belgium and the Netherlands (Van Knapen, 1983). The same situation exists in endemic regions of Africa (Walther and Koske, 1980).

It would be unrealistic to contemplate that improvement in the already established meat inspection practices would significantly increase the reliability of this diagnostic procedure in bovine cysticercosis. An approach based on serological detection of the infection would therefore appear to be a practical possibility. The success of this approach, however, depends entirely on whether it is possible to develop a reliable, sensitive and specific immunological assay which preferably can be adapted to slaughterhouse procedures. This approach has already been successfully applied to trichinosis in pigs (Ruitenberg, 1977; Ruitenberg et al., 1977).

While sensitive methods are available, two major obstacles remain unsolved in the case of bovine cysticercosis, namely, the relatively poor immunological response of naturally infected animals, and cross reactions due to antigens shared with other common parasites of cattle (Gathuma and Waiyaki, 1980, 1981; Geerts et al., 1981a,b,c.; Kamanga-Sollo and Lindqvist, 1982).

As a consequence of the considerations outlined above the aims of this study were to:

(i) Define the antigenic components of T. saginata metacestode

(ii) Investigate the relationship between these antigens and those of other parasites.

(iii) Select antigens specific for the metacestode or antigens which share determinants with few parasites.

(iv) Isolate and characterize the selected antigens for use in serological tests.

(v) Examine the specificity of these antigens using highly sensitive methods such as enzyme immunoassays and radioimmunoassay.

2. GENERAL REVIEW OF LITERATURE

The literature dealing with human taeniasis and bovine cysticercosis is extensive, as exemplified by the list of 1625 references compiled by Doby and Herisset (1979) covering the period up to 1979. Several review articles have also been published (Pawlowski and Schultz, 1972; Gemmell and MacNamara, 1972; Gemmell and Johnstone, 1977; Williams, 1979; Rickard and Williams, 1982).

In the following general review of the literature, only the general aspects will be covered, while the literature pertaining to the specific objectives of this study will be reviewed in the appropriate chapters.

2.1 Distribution of *T. saginata* cysticercosis and taeniasis

Taenia saginata infection in man and bovine cysticercosis in cattle are manifestations of an important zoonosis which represents a severe economic burden on the livestock industry in many parts of the world.

Epidemiological data on the prevalence of human taeniasis (*T. saginata*) is grossly defective and inadequate. Frequently, only a portion of the population is examined, for example, children, hospital patients, or the number of people examined is too small to give a true picture of the prevalence. However, a conservative estimate gives the number of infected persons in the world to be about 80 million, of whom 32 million live in Africa (Beir, 1965; Frolova, 1982).

In hyperendemic areas of Africa 10-50% of the human population may be infected with T. saginata (Frolova, 1982). The highest prevalence rate (50%) of human taeniasis has been reported in Ethiopia (Frolova, 1982). In other beef ranching areas such as Kenya, Botswana, Tanzania, Sudan, Zaire and South Africa, prevalence rates of more than 10% have been recorded (Van Grunderbeeck and Penson, 1954; Elsdon-Dew, 1964; Froyd, 1965; Hoeppli, 1969). In contrast, low prevalence rates have been recorded in the forest regions of Cameroun (Doby et al., 1957) and in the ethnic groups inhabiting the coastal areas of Kenya (Froyd, 1965). No infection has been found in the pygmies in Zaire (Price et al., 1963).

In Asia, taeniasis is widespread in Mongolia, Campuchea and Japan, where 20-30% of the population in some villages may be infected (Frolova, 1982). In South and Central America the prevalence is high, as exemplified by Argentina, Brazil, Columbia, Mexico, Guatemala, Uruguay and Venezuela, where up to 3% of the population may be infected (Frolova, 1982).

Human taeniasis occurs in almost all European countries, although it is less frequent in comparison with other parts of the world (Pawlowski, 1971; Frolova, 1982). There has been, however, an increase in its prevalence in some countries. For example, in the German Democratic Republic, an increase from 1.5% to 4.5% has been noted during the period 1966-1973 (Muller, 1970; Hiepe and Buchwalder, 1978).

Taeniasis is also widespread in the Soviet Union, where an average infection rate for the whole country is 0.02%

(Frolova, 1982). The highest prevalence (43.5%) of human infection has been registered in Uzbek, SSR (Magdiev, 1968; Magdiev, 1982).

In the United States of America, the endemic areas of the South Western United States have maintained a constant level of about 1% (Schultz et al., 1970).

Bovine cysticercosis shows a distribution pattern similar to human taeniasis. It is estimated that more than 23 million head of cattle are infected with T. saginata cysticercosis in Africa (Mann, 1983). In Europe, a high prevalence rate of bovine cysticercosis is recorded in the German Democratic Republic where more than 3% of slaughtered cattle harbour cysticerci (Hiepe and Buchwalder, 1978). A study carried out in USA by Schultz and his coworkers (1970) revealed annual figures of T. saginata cysticercosis ranging from 12,000 to 16,000. The prevalence of bovine cysticercosis for the whole of the USSR is estimated at 1% (Pawlowski and Schultz, 1972). However, in some areas of Armenia the rate is as high as 20.7% (Avakyan, 1961).

2.2 Hosts of the adult worm and the larval stages

The only definitive host for the adult stage is man, many of the laboratory animals failed to develop adult T. saginata infection when fed with intermediary stage (Nelson et al., 1965). An extensive survey carried out in Kenya involving more than 271 wild primates revealed 6 different species of tapeworms, but none was identified as

T. saginata (Nelson et al., 1965). In general, cestode larvae have limited host specificity. The main intermediate hosts for T. saginata metacestode are domesticated bovidae which include Bos taurus, Bos bufellus, Bos indicus and Bos grunniens (Abuladze, 1964). Other identified hosts, include reindeer (Rangifer tarandus). Reindeer meat has been implicated as a source of taeniasis in many regions of USSR, where cattle are not raised (Krotov, 1961; Abuladze, 1964).

In the Americas, the only implicated wild animal intermediary host for the T. saginata, cysticerci are the llama (Lama glama) and pronghorn antelope (Antilocapra americana). In Africa, however, cysticerci without rostellar hooks, presumably the metacestode of T. saginata, have been described in a number of animals which include wildebeest (Connochaetes taurinus), bushbuck (Tregelaphus scriptus), oribi (Ourebia ourebi), Dorcas gazelle (Gazella dorca), Corrina gazelle (Gazella rufrifrons), Thomson gazelle (Gazella thomsonii), topi (Damaiscus korrigum), oryx (Oryx spp) and giraffe (Giraffa camelopardalis) (Nelson et al., 1965; Graber, 1959, 1974; Stevenson et al., 1982). This would suggest that the introduction of ranching and cropping of wild ruminants to provide meat for human consumption may become an important factor in the dissemination of taeniasis and T. saginata metacestodiasis.

Huang (1967) found 28 tapeworms which were identified as T. saginata in people of Wulai District of Taiwan. However, there are no cattle in this area. Goats, therefore, have been

suspected as natural hosts in this region. There will probably never be a final list of all intermediate hosts of T. saginata (Pawlowski and Schultz, 1972).

One of the most controversial questions about the intermediate hosts is the role of man himself. Two different opinions have been expressed concerning cysticercosis of man. Nelson et al (1965) have suggested that some cysts reported in man may be cysts of T. saginata or small hydatid cysts. On the other hand, Verster (1967) commented on the discrepancy between the incidence of human cysticercosis and that of adult T. solium infection. She indicated that T. solium infection may not be as rare as generally assumed.

There is no doubt that cysticerci with hookless scolices have been found in man (Pawlowski and Schultz, 1972). There are instances where individuals harbouring T. saginata may also have concurrent cysticercosis (Fontan, 1919; Naumova, 1955). According to Pawlowski and Schultz (1972), twelve cases of T. saginata cysticercosis have been described in the literature. These include cases described by Meggit (1924), Brumpt (1936); Castellano et al. (1928); Fontan (1919); Naumova (1955); de Rivas (1937); Tanasescu and Repciuc (1939); Asenjo and Bustamente (1950); Nino (1950); Bacigalupo and Bacigalupo (1956); Abuladze (1964) and Goldsmid (1966). A characteristic case, described by Tanasescu and Repciuc (1939), was that of a 59 year old Rumanian woman with a tumour in the mammary region. Their article contained seven photographs of sections through an unarmed scolex. They believed it was a cysticercus

of T. saginata because it had five suckers instead of the four which are characteristic of T. solium. Since the photographs also showed rugae in the wall surface, the diagnosis is probably correct.

2.3 Transmission between man and animals

T. saginata infection is a true anthropozoonotic helminthiasis (Sprent, 1969a,b) where man is the essential definitive host and disseminator of the infection, while cattle serve as the most important intermediate hosts. Transmission from man to animal may either be direct or indirect.

Direct transmission may occur when hands contaminated with T. saginata eggs, feed or handle calves (Urquhart, 1961; Goulart et al., 1966). However, the indirect route is the usual one, and occurs through contamination of cattle feed and pastures by human faeces and sewage. Birds, flies and wind may contribute to the spread of eggs. Wherever there is a close contact between infected humans and susceptible animals, heavy infection results, for example in feedlots, where large-scale fattening is practised (Frolova, 1982). On the other hand, light infection results when eggs are widely distributed in the environment.

A variety of ecological factors influence the viability of eggs. Taeniid eggs will withstand the action of unfavourable external factors quite well. The eggs can survive the action of most chemical disinfectants (Laws, 1967; Mackie and Parnell, 1967). They also resist a variety of physical

factors (Laws, 1968; Buchwalder and Hiepe, 1982). According to Laws (1968), dessication is an important factor affecting the survival of taeniid eggs. Under natural conditions, temperature and moisture are generally accepted as the most important factors involved in the survival of T. saginata eggs (Penfold et al., 1937; Jepsen and Roth, 1952; Silverman, 1956b; Suvorov, 1965). At temperatures of 4-5°C, T. saginata eggs can survive for at least 168 days (Froyd, 1962). According to Suvorov (1965), the optimal temperature for survival of T. saginata eggs is -40°C. Jepsen and Roth (1952) found that T. saginata eggs survived at least for 16 days when stored at 18°C in a dish filled with liquid manure. However, Duthy and Van Someran (1948) found that eggs could survive for about a year in pastures of the highlands of Kenya. Eggs survive better when they are free than when they are within the proglottids (Suvorov, 1965).

Sewage is an important source of dissemination of T. saginata eggs (Profe, 1934; Sinnecker, 1955; Liebman, 1963; Crewe, 1984; Collier and Reilly, 1984). The increase of urban populations with consequent increased usage of water and detergents and overloading of sewage works, lead to breakdown of formerly reliable sewage treatment systems and survival of T. saginata eggs in the effluent and sludge from sewage plants (Silverman and Griffiths, 1955b; Muller, 1970; Arkhipova, 1977; Hammerberg et al., 1978; Mann, 1983). The increased use of detergents interferes with the sedimentation and oxidation processes and enables a greater portion of parasite eggs to

survive these processes (Silverman and Griffiths 1955b). Improperly treated sewage effluent, as a source of infection to cattle, has been noted in a variety of circumstances. For example, in the United Kingdom, Silverman (1955a,b) stated that 50% of cattle drank sewage-polluted water. In USA, Miller (1956) commented that changing farming and irrigation practices increased the likelihood for livestock to be exposed to contaminated sewage. Epidemics of T. saginata infection have been attributed to sewage (Schultz et al., 1969). Sinnecker (1955); Liebman (1963); and Denecke (1966) have suggested that there is an inverse correlation between the efficiency of sewage treatment and spread of cysticercosis in some areas of Europe. These observations have lead to the conclusion that sewage effluent should be used with caution in agriculture (Miller, 1956; Greenberg and Dean, 1958; Muller, 1970).

Dissemination of eggs by birds has been suggested, implicating mainly the gulls and other birds which feed on proglottids (Gotzche, 1951; Silverman and Griffiths, 1955b; Guidal, 1956; Crewe, 1967; Crewe and Crewe, 1969). Guidal (1956) found eggs of Taenia species in the intestinal tract of blackhead gulls (Larus ridibundus), common gulls (L. canus), but none in herring gulls (L. argentatus) or lesser blackheaded gull (L. fuscus).

Shircore (1916) discussed the transmission of helminth eggs by flies. In Uzbek, SSR, Sycevskaja and Petrova (1958) observed that flies transmitted T. saginata eggs. Round (1961) examined filth flies (Chrysomyia albicans, Ch. cloropyga and

Sarcophaga species) in Kenya. He stated that T. saginata eggs can be passed by these flies for a period of up to 11 days after ingestion. This observation supports the conclusion that filth flies play an important role in the transmission of Taenia eggs. According to Nadzhafov (1967) in Azerbaijan, SSR, which is an endemic area of T.saginata infection, 4.8% of 4372 flies belonging to 20 species had eggs of Taenia species on their external surfaces or in their intestinal tracts. The highest infection rate (8.8%) was observed in Sarcophagidae. On the basis of these observations, Nadzhafov (1967) concluded that synanthropic flies play a definite role in the dissemination of T.saginata infection.

Since man is the only known definitive host of T.saginata, the only geographical limitation of this infection is the regions of the world inhabited by man. Ecological factors have an important role in determining the cattle breeding areas of the world. But wild animal reservoirs of this infection have been found in Taiwan (Huang, 1967), and the northern zone of the USSR (Abuladze, 1964; Krotov, 1961). The role of wild animal reservoirs in Africa is not clear (Nelson et al., 1965).

Transmission between animals and man also depends on ethnological factors, human habits, behaviour, religion and beliefs. These influence the type of food man consumes and the manner of its preparation. Some practices are based on hundreds of years of tradition, some of these are condusive to the transmission of T. saginata and it is doubtful that they

can be changed rapidly, (Mann and Mann, 1947; Abasöv, 1957; Carmichael et al., 1952; Kovalev, 1965; Abdullaev, 1968; Pawlowski, 1970; Frolova, 1982; Mann, 1983). Studies of the social and ethnological aspects of taeniasis are obviously important integral part of epidemiological investigations of T. saginata zoonosis.

2.4 Losses due to taeniasis and cysticercosis

Economic losses resulting from the cost of freezing, downgrading of conditionally passed meat and total condemnation of heavily infected carcasses are substantial (Urquhart, 1958; Mango, 1971; Taylor, 1975; Gathuma, 1979; Mann, 1983). The medical costs of human T. saginata taeniasis are difficult to establish since the infection is usually asymptomatic. Symptomatology is quite varied and probably causes some lowering of productivity in the infected population (Mann, 1983). The combined effects in a population living on a protein deficient diet and loaded with other parasite, should be considered (Mann, 1971).

Considerable information is available about the financial losses due to T. saginata cysticercosis. The annual economic losses caused to livestock production in the world is estimated to be in the range of hundreds of millions US dollars (Bessonov and Krotov, 1982). Table 1 summarizes the estimated losses in different parts of the world. These figures are impressive but cannot be taken as valid for other regions. A better way is to estimate the average loss per animal and multiply it by the

number of infected animals for each particular region. According to Logan (1967) the mean loss per infected animal in the world is estimated to be Sterling Pound 10. Silverman and Griffiths (1955a) ascribed the major losses to decreased value of the meat, loss of weight (about 2%) and increased costs of additional handling, refrigeration and transport. These authors estimated a mean loss of Sterling Pound 30 per animal in the United Kingdom. In USA, the cost of freezing an infected carcass is approximately US $ 75 and a condemned carcass costs approximately US $ 300. Thus a reasonable estimate of the loss due to cysticercosis is US $ 25 per animal in developing countries, and US$75 in industrialized countries (Pawlowski and Schultz, 1972). In the 10 year period since these estimates were made, the losses are expected to have trebled. In general, an infected carcass is worth one third less than a non-infected animal (Costa and Brant, 1964).

Apart from the direct losses, there are other important consequences of bovine cysticercosis. This is especially true in developing countries which are seeking to develop a profitable beef industry (Paswlowski and Schultz, 1972). For instance, the European Economic Community (E.E.C.) directive 72/452 of December 1982 states that "Meat originating from animals carrying one or more live or dead cysticerci shall not be exported to E.E.C. Countries" makes the operation of an export oriented meat industry very difficult in areas where the prevalence of bovine cysticercosis is high and prohibits earning of essential foreign exchange (Mann, 1983).

2.5 Immune responses to cestodes

The presence of natural resistance to cestode parasites has been proposed by many workers (Gemmell and MacNamara, 1972; Barriga, 1981). Cestodes, especially the adult stages, possess remarkable specificities for their hosts. The whole group of cestodes are restricted to particular orders of mammals or birds and many individual tapeworms will develop only in a single definitive host species. As a general rule, the larvae appear to enjoy a broader specificity than the corresponding adults. Many larval cestodes will develop in unsuitable hosts when oncospheres are artificially activated and injected parenterally (Barriga, 1981). To some extent, this situation must be expected, because the difference in the instestinal environment among species of mammals must be more marked than the variations in their milieux interieur (Barriga, 1981).

For many years it was considered unlikely that intestinal infection with adult cestodes could provoke immunological responses in the definitive hosts. The superficialty of contact between the parasite and the tissues of the host was the basis for this argument. This notion was supported by clinical observations that, left untreated, the intestinal cestodiases of humans persisted for a number of years. It seems obvious that an immunological rejection mechanism to T.saginata and T. solium infection is poorly developed (Williams, 1979; Barriga, 1981). However, circulating antibodies to adult cestodes have been demonstrated in experimentally infected animals. For example, in Hymenolepis

diminuta infections, antibodies have been detected by employing tests such as complement fixation, indirect fluorescent antibody and passive cutaneous tests (Coleman et al., 1968; Harris and Turton, 1973). Skin sensitizing antibodies have also been described in dogs naturally infected with Echinococcus granulosus (Williams and Perez-Esandi, 1971). Haemagglutinating antibodies were present in sera from patients infected with T. saginata (Machnicka, 1974b). Nepote et al., (1974) observed an increase in the level of IgE in patients infected with T. saginata. The level of reagenic antibodies decreased to normal levels after treatment of patients with niclosamide. Elevated levels of IgE have been reported in humans infected with other helminths (Johannson et al., 1968). The ability of helminths to stimulate reaginic antibodies in experimental animals have also been shown by Sadun (1972). Skin sensitizing antibodies have also been detected in sera of rats and mice infected with T. taeniaeformis and in rabbits infected with T. pisiformis (Musoke and Williams 1975b; Leid and Williams, 1974b, 1975). Generally, reagenic antibodies in rats and rabbits appeared in the circulation within the first 2 to 3 weeks, reached a plateau over the subsequent 2 to 3 weeks and then declined. On oral challenge with eggs, a secondary response was observed (Williams, 1979).

The role that circulating antibodies play in regulating tapeworm growth or rejection is not clear, because there has been no successful demonstration of passive transfer of protection with serum (Williams, 1979). Local antibody

production may be important. Support for this possibility arose from the detection of immunoglobulins and component C3 of the complement system on the tegument of H. diminuta in mice (Befus, 1974), and demonstration of membrane lesions that may be the result of antibody/complement mediated damage (Befus and Threadgold, 1975). The significance of these observations is still difficult to assess because immunoglobulins and C3 have also been demonstrated on the surface of H. diminuta in rats in which the worms survive for prolonged periods in the intestinal lumen (Befus, 1974).

Epidemiological studies on naturally occurring cysticercosis in domesticated ruminants have implicated acquired immunity as an important determinant of infection in animal populations (Gemmell and Johnstone, 1977; Williams, 1979). During the 1960's there was experimental verification of protective response to challenge infection with T. saginata in cattle (Urquhart, 1961) and with T. ovis and T. hydatigena in sheep (Blundell et al., 1968; Gemmell, 1970; Wilkerhauser et al., 1974). Immunization with killed organisms or antigenic extracts was consistently ineffective, giving rise to the notion that immunity could be induced only by the use of live parasites (Williams, 1979). Research workers in Australia have, however, shown that successful vaccination of animals is possible if excretory-secretory products of cestodes are used. In this regard, Rickard and Bell (1971a) were able to provoke a high degree of resistance to T. ovis in lambs by implanting intraperitoneal diffusion chambers containing activated

oncospheres. Vaccination with antigens collected from 14 day *in vitro* culture of embryos of *T. ovis* was also effective (Rickard and Bell, 1971b). Rickard *et al.*, (1976, 1977b) have also demonstrated that vaccination of 4-month old lambs with antigens obtained from *in vitro* cultures resulted in immunity to challenge with *T. ovis* eggs. Vaccination of pregnant ewes resulted in colostral transmission of resistance to lambs (Rickard *et al.*, 1977b). In parallel experiments, Rickard and Adolph (1976) immunized calves with antigens collected from 14-day *in vitro* cultures of *T. saginata*. They showed that vaccinated calves were highly resistant to homologous challenge. Calves which received colostrum from cows which had been similarily vaccinated, were also shown to be highly resistant to *T. saginata* infection (Rickard *et al.*, 1977a). Successful vertical transmission of immunity to offspring strongly suggests that antibodies are involved.

The antibody mediated protective mechanism is a cardinal feature of immunity to *T. taeniaformis* in the mouse and rat, and to *T. pisiformis* in rabbits. Protective antibodies in rats infected with *T. taeniaeformis* are exclusively associated with immunoglobulin of the IgG_{2a} class during the first 4 weeks of infection (Leid and Williams, 1974a). Thereafter, chromatographic fractions containing other immunoglobulins are effective in passive transfer (Musoke and Williams, 1975a). The form in which the immunogens are presented to the experimental animals appear to be important, because protective antibodies produced in response to intraperitoneal implantation

of worms differ markedly from those which develop during natural infection (Musoke and Williams, 1976). IgA antibodies are known to be involved in natural passive transfer of resistance from mother rats to their young (Musoke et al., 1975). These antibodies function at the intestinal level (Hammerberg et al., 1977). In passive transfer experiments in mice, protection was associated with chromatographic fractions enriched for IgG1 (Musoke and Williams, 1975b) although colostral IgA has been implicated in passive protection of neonatal mice against T. taeniaformis (Lloyd and Soulsby, 1978). There is a coincidental development of IgG2a and IgE antibodies to T. taeniaformis in the rat. This led to speculation that reagins might play a role in the protection mechanism (Musoke et al., 1978). Passively transferred antibodies appeared to be most effective during the early developmental stages of T. taeniaformis in the rat liver. After 5 days the parasite rapidly becomes invulnerable to antibody mediated attack (Campbell, 1938; Musoke and Williams, 1975a; Mitchell et al., 1977). Depletion of circulating complement in passively immunized rats over the initial 5 day period of growth of the parasite, led to a very significant increase in worm survival (Musoke and Williams, 1975a). This observation implicated the involvement of complement in the effector mechanism.

Siebert et al. (1978) concluded that complement-mediated destruction of the larvae of T. crassiceps occurred in immune mice. These results suggested that parasites which survive in

the immune host must be able to avoid the combined effects of complement and antibody. Several hypotheses have been advanced to account for the prolonged survival of cestode parasites in immunologically competent animals. These include membrane fixation of blocking antibodies which could interfere sterically with the attachment of protective antibodies (Varela-Diaz et al., 1972) or with the attachment of specifically sensitized cells (Rickard, 1974; Kwa and Liew, 1978).

Table 1. Estimated annual losses due to bovine cysticercosis in different parts of the world.

Countries	Estimated loss	References
Federal Republic of Germany	5 million DM	Friedrich, (1961)
German Democractic Republic	121000 DM	Mielke, (1969)
Yugoslavia	6 million dinars	Winterhalter, (1965)
Czechoslovakia	2.6 million Czech Cr	Bartak et al., (1982)
Belgium	25 million francs	Granville et al., (1958)
United Kingdom	100,00-500,000 St	Silverman & Griffiths, (1955a)
Denmark	1-3 million Kroner	
France	30 million francs	Bessnov & Krotov,
Latin America	39 million American dollars	(1982)
Kenya/Uganda/Tanzania	20 million St	Taylor, (1975)

CHAPTER I

THE ANTIGENS OF T. SAGINATA METACESTODE AS DEFINED BY CROSSED IMMUNOELECTROPHORESIS.

1. INTRODUCTION AND REVIEW OF LITERATURE

For reasons of procedural convenience, the antigenic preparations used by many parasitologists in their quest for a serodiagnostic method for bovine cysticercosis have been crude extracts of T. saginata (Van Knapen et al., 1979a; Harrison and Sewell, 1981a). The ubiquity of common antigens among a large number of parasites is a major obstacle to achieve specific reactions with these reagents. However, the presence of antigens which are shared by various developmental stages of the same parasite may have obvious advantages in that positive serological reactions may be obtained in different clinico-biological phases of the infection (Barriga, 1981).

There are a few reports on the analysis of the composition of T. saginata (Biguet et al., 1965; Capron et al., 1968: Geerts et al., 1979). However, excellent studies have been reported on the isolation and characterisation of helminth antigens for use in immunodiagnostic tests (Kent, 1963; Kagan and Norman, 1963). One of the initial analysis of helminth antigens was made with Trichinella spiralis (Wodehouse, 1956). In his analysis of larval extracts, Wodehouse (1956) demonstrated ten precipitin bands using the and Ouchterlony (1948) Oudin (1952) immunodiffusion techniques. Using the Oakley and Fulthorpe (1953) techniques, Kagan and Bargai (1956) found three major bands produced by a larval extract of T. spiralis prepared by the method of Melcher (1943) and antisera obtained by

experimental infection with T. spiralis in rabbits. Further studies using immunoelectrophoresis revealed 6 to 7 bands in larval antigenic extracts and two bands with adult parasite extracts (Oliver-Gonzalez and Levine 1962; Oliver-Gonzalez, 1963). The apparent discrepancy in the number of bands obtained by Woodhouse (1956) and other workers, was primarily due to the type of antisera used. While Woodhouse (1956) analysed his antigens against antisera obtained in rabbits by immunization, Kagan and Bargai (1956), and Oliver-Gonzalez and Levine, (1962) analysed antisera obtained by infection. One generally finds fewer antigen-antibody reactions with sera from infected animals as compared to sera produced by immunization (Barriga, 1981). However, by using more sensitive methods, such as crossed-immunoelectrophoresis or immunoelectrophoresis, additional antigenic components may be demonstrated (Tanner and Gregory, 1961; Geerts et al., 1979). Other parasitic antigens have been assayed for complexity by agar-gel diffusion methods. Soulsby (1957) analysed the antigens of Ascaris lumbricoides suum and reported that the intestinal tissue of this parasite produced 14 bands, eight of which could be removed by absorption with sheep erythrocytes. He believed the latter to be Forssman antigens.

In their studies, Geerts et al. (1979) analysed the antigenic structure of T. saginata using the Grabar and Williams (1953) immunoelectrophoresis technique. They described 22 precipitating components. By incorporating host antigens from bovine liver and serum, and heterologous helminth

antigens in cross absorption experiments, they found that only seven antigens were specific for T. saginata cysticerci. These findings are similar to those described by Capron et al. (1968) who reported that two thirds of the 23 components of T. saginata were shared by other cestodes.

Crossed immunoelectrophoresis is a powerful analytical tool for the analysis of antigenic composition of infective agents (Roberts et al., 1972; Closs et al., 1975; Huppert et al., 1978). Antigens which are common to various parasites can easily be compared using this method.

The purpose of the study presented in this chapter was to establish a reference pattern for the antigens of T. saginata metacestodes. This system was further used to define, select and analyze antigens which were unique to T. saginata metacestodes and to elucidate the extent of crossreactions by identifying those antigens which were shared with other parasites.

MATERIALS AND METHODS

2.0 ANTIGEN PREPARATIONS

2.1. *T. saginata* metacestodes

T. saginata metacestodes were obtained from infected meat bought from various slaughterhouses in Nairobi and the Kenya Meat Commission (KMC) slaughter house in Athi River. The meat was placed in plastic bags and delivered to the laboratory where cysts were carefully dissected from it. The outer connective tissue capsule which is derived from host components (Thornton, 1968) was carefully removed by dissection and referred to as outer membrane (OM). The intact cyst bladders were washed with saline, blotted dry and placed in petridish. The bladder was then punctured using a dissecting needle and the fluid obtained from the bladder was harvested and termed the inner fluid (F). The fluid of a number of metacestodes was pooled and sodium azide (BDH-Chemicals Poole, England) added to a concentration of 0.1%. One portion of the fluid was lyophilized and stored as a dry powder, while the other portion was stored frozen at -20°C.

The bladder and scolex was washed several times with saline and dried on filter paper. The bladder and the scolex were termed the "Inner Membrane Scolex" (IMS). This was divided into batches of 10g (wet weight) and 0.1% sodium azide added. The preparation was stored frozen at -20°C.

Crude saline extracts from OM and IMS were prepared following the procedure described by Kamanga-Sollo (1981). Briefly, the method involved the following steps. Ten grams

wet weight of either frozen OM or IMS were placed in mortars cooled to -20°C and ground using a cooled pestle. Ten mls of saline were added and the material further homogenized using a Potter-Elvehjem tissue grinder (Kontes-Glass Company, New Jersey, USA) in ice water. The homogenate was then sonicated at 300 watts for 5 minutes in an ice-water bath using the Brausonic 1510 sonicator (Braun Melssungen AG, Germany). Aliquots of the sonicated material were used for the production of antisera in rabbits (see section 2.6.2), while the rest of the material was centrifuged at 3000xg in a refrigerated centrifuge at 4°C (Beckman-Model J218 centrifuge, Beckman Instrument Inc., California, USA). The supernate was divided into aliquots which were lyophilized and stored as a dry powder at -20°C.

2.2. T. saginata

2.2.1 Collection of T. saginata tapeworms

Children in various Masai "Manyatta" (homesteads) and from primary schools in Kajiado District, were treated with Yomesan (2,5-dichloro-4-nitrosalicylanilide) followed by magnesium sulfate as cathartic. Whole tapeworms and segments were recovered from the stools. Tapeworm segments were washed clean of intestinal contents with water and the proglottids stored at 4°C in saline containing 0.2% sodium azide.

2.2.2 Crude saline extracts of the whole worm

The tapeworm segments were rinsed with saline. Preparation of crude saline extract was performed according to the method described by Kamanga-Sollo (1981). Briefly, the following protocol was followed. Clean tapeworm segments weighing 5g (wet weight) were homogenized in a tissue blender (Measuring and Scientific Equipment, England) placed in an ice-water bath. Ten mls of saline were added and the homogenate further homogenized using a Potter-Elvehjem tissue grinder placed in an ice-water bath. The homogenate obtained was sonicated at 300 watts for 5 minutes in ice water bath using the Brausonic 1510 sonicator.

A portion of the homogenate obtained after sonication was used for the production of antisera (see section 2.6.2) in rabbits.

Another portion of the sonicated homogenate was centrifuged in a refrigerated centrifuge. The supernate was harvested and divided into aliquots, which were lyophilized. The antigen extracts were stored as freeze dried powder at $-20^{o}C$.

2.2.3 T. saginata eggs

Eggs of T. saginata were obtained from mature proglottids. The eggs were teased out using dissecting needles and divided into two batches. One batch was used in experimental infection of calves (see Chapter III), while the other batch was used for comparison with the cyst antigens.

This batch was processed as described for T. saginata whole worm antigens.

2.3 Other parasites

Parasites common to various domestic animals were obtained from various slaughter houses around Nairobi and in Kajiado District, while parasites from wildlife were obtained from animals at the Masai Mara Buffalo Camp.

The list of parasites obtained is given in Appendix I. The parasites were washed with water, and placed in universal bottles containing saline with 0.1% sodium azide. Extraction of parasite antigens was performed essentially as described in 2.2.2. for Taenia antigens, while fluid from metacestodes was harvested and treated as described in 2.1.1.

2.4 Parasites and antigens obtained from other laboratories

T. solium metacestode antigens were obtained from Dr. A. Flisser of the University of Mexico, as a freeze dried potassium chloride extract.

Schistosoma mansoni extract was obtained from Dr. Z. Ahmed of the Department of Human Pathology, University of Nairobi.

T. saginata oncospheres were obtained from Mrs. J. Onyango-Abuje of the Kenya Agricultural Research Institute, Muguga.

2.5 Antigens used in immunodiffusion and immunoelectrophoresis analysis

For the use in crossed immunoelectrophoresis and immunodiffusion analyses, all antigen preparations were reconstituted to make a final concentration of 10% dry weight/volume in distilled water, Triton X-100 added to a concentration of 1%, and sonicated at 300 watts for 3 minutes in an ice-water bath using a Brausonic 1510 sonicator. The protein content of all antigens was determined following the method described by Lowry et al. (1951). The protein contents are given in Appendix I.

2.6 Antisera

2.6.1 Experimental animals

Antisera used in this study were prepared in rabbits and goats. New Zealand large white rabbits were purchased from the Veterinary Laboratories, Kabete, and the National Public Health Laboratories. Local breeds of goats were purchased from farmers in the Ongata Rongai area in Kajiado District.

The rabbits were treated for coccidiosis using Amprol^{R} (Merck-Sharp Dohme). They were kept in isolation and fed on concentrates (Rabbit pellets, Unga Ltd, Nairobi), and provided with water ad libitum. The animals were bled before commencement of the immunization schedule.

The goats were treated with anthelmintic (Thialbenzole, Merck-Sharp Dohme) before being placed in isolation and fed on

hay and concentrates (Ewe and Lambnuts, Unga Ltd., Nairobi). They were provided with water and salt lick (Wellcome Laboratories, Nairobi) ad libitum.

2.6.2 Production of antisera

The list of antisera prepared is given in Appendix II. Other batches of antisera were available from a previous study conducted by the author.

The first dose of each antigen was emulsified in Freund's complete adjuvant (DIFCO Laboratories, Detroit, USA) mixed in a volume ratio of 1:3.

Emulsified antigen was injected into lymph nodes also according to the method described by Newbould (1965), and injected intramuscularly. Subsequent booster doses were emulsified in Freund's incomplete adjuvant (DIFCO Laboratories Detroit USA) and injected intramuscularly.

Immunization and test bleedings were done at two weeks, intervals until a maximum number of precipitin lines were observed when tested against homologous antigen extract in Ouchterlony's double diffusion method and with the cross-bleeding immunolectrophoresis techniques. The immunization and bleeding schedule continued in some cases for a period of 24 months. The preparation of specific antisera to various components of T. saginata metacestodes is describe in 2.6.3.

Batches of different antisera were prepared by pooling several bleedings from each animal immunized with the same batch of antigen preparation.

2.6.3 Preparation of specific antisera to components of *T. saginata* metacestode antigen (IMS)

Antisera specific for various defined antigens can be obtained by immunization with washed immunoprecipitates obtained either by immunoelectrophoresis or crossed immunoelectrophoresis (Goudie *et al.*, 1966; Harboe *et al.*,1976).

In this study, several specific antisera to antigenic components of IMS were produced by immunization of rabbits using immunoprecipitates obtained from immunodiffusion tests using IMS as antigens and pooled rabbit anti IMS (Appendix III). These components were defined and given numbers in crossed immunoelectrophoresis.

2.6.4 Preparation of precipitin lines for immunization of experimental animals

A number of microscope slides were prepared for immunodiffusion (see section 2.7.1). The diffusion was allowed to proceed for 18 hours at room temperature, after which the precipitin lines were clearly visible. The slides were washed as described in 2.7.2 but they were not dried or stained. The precipitin bands were cut out of the gel using razor blades as close as possible to the line. The agar strips containing the precipitin lines were washed at $4^{o}C$ for five days using large volumes of phosphate buffered saline (PBS), pH 7.4, containing 0.1% sodium azide. There were several changes of buffer.

For each immunization, precipitin strips obtained from approximately 100 immunodiffusion slides were used. The PBS was sucked off and strips sonicated in an ice bath using a Brausonic 1510 sonicator with an effect of 100 watts in 1 second pulses until the agar had visibly been dispersed. Freund's complete adjuvant was added in a ratio of 1:3 and the mixture sonicated till it became a thick, stable paste. The subsequent booster injections were performed as described, except that Freund's incomplete adjuvant was used.

2.6.5 Harvesting and storage of the antisera

Freshly drawn blood was allowed to clot by standing overnight at room temperature. The clot was loosened from the walls of the siliconized tubes using an applicator stick, the serum decanted and centrifuged at 3000 rpm for 15 minutes in a refrigerated centrifuge (Minifuge 2, Heraeus Christ F.R. Germany) to separate the serum. Sodium azide was added to a final concentration of 0.1% and the sera stored frozen at $-20^{o}C$.

2.6.6 Absorption of antisera with insoluble immunoadsorbents

Insoluble immunoadsorbents were prepared by coupling the antigen(s) desired for absorption to CNBr-Sepharose 4B (Pharmacia Fine Chemicals Co., Uppsala, Sweden). The immunoadsorbents were prepared following the manufacturer's recommendation with slight modifications. Briefly, the method was as follows: The Sepharose was washed with 1 mM HCl in a

ratio of 200 mls per gram of dry Sepharose. This was followed by rinsing with a copious amount of distilled water. The antigen preparation to be coupled was dissolved in coupling buffer (0.1M sodium bicarbonate buffer, pH 8.3, containing 0.5M sodium chloride) and mixed with the gel suspension in an end-over-end mixer (Voss of Maldon, Essex, England) for 2 hours at room temperature and for 18 hours at 4°C. The uncoupled components were washed off with coupling buffer and the remaining active groups blocked by 1M ethanolamine at pH 9.6 for 2 hours at room temperature. The blocking agent was washed off using coupling buffer followed by 0.1M acetate buffer pH 4.0 containing 0.5M sodium chloride and finally rinsed with coupling buffer and saline.

The uncoupled protein content was determined according to the method of Lowry et al. (1951) to allow calculation of the coupling efficiency. With serum proteins as antigens to be coupled, approximately 80% was coupled to Sepharose. The antigen-Sepharose immunosorbent was stored at 4°C as a 50% suspension in saline with 0.1% sodium azide.

2.6.7 Procedure for absorption of antisera using insoluble immunoadsorbents

The absorption of antisera was performed by mixing the immunosorbent with antiserum in an end-over-end rotator at room temperature for 2 hours and in the cold (4°C) for 18 hours.

The immunosorbent was then separated from serum using a sintered glass filter (G3-Pyrex England). The immunosorbent

was regenerated by washing with 0.2M glycine/HCl buffer, pH 2.8, followed by coupling buffer and saline.

This absorption procedure was repeated, until appropriate tests had shown that the antiserum possessed the required specificity.

2.6.8 Antisera obtained from other laboratories and coworkers

Antisera to Fraction 10 of T. saginata was obtained from Dr. Geerts of the Institute of Tropical Medicine, Belgium.

Other antisera, shown in Appendix II, were obtained from colleagues who were working on different projects in the Department of Public Health, Pharmacology and Toxicology, Faculty of Veterinary Medicine, University of Nairobi.

2.7. Analysis of the antigens

2.7.1 Immunodiffusion tests

The microtechnique of Ouchterlony's double diffusion in agar described by Crowle (1973) was used with slight modifications. Briefly, the method was as follows: One percent (W/V) of purified Oxoid agar (Oxoid Ltd, England) in PBS with 0.1% sodium azide was used in the preparation of the gel for double diffusion. Three millitres of molten agar was poured on the microscope slide (25 x 75mm) to give a depth of approximately 2mm. With a gel-puncher (Gelman Instruments Co., Ann Arbor, Michigan, USA) wells of 4mm diameter and 5 mm apart

were cut in hexagonal patterns with central wells. The agar in the wells was removed by suction. The central well was filled with 15 ul of antiserum while peripheral wells contained 15 ul of antigen preparations. This pattern was sometimes changed by placing antigen in the central well and antisera in the peripheral wells.

The slides were placed in a humid box and diffusion allowed to proceed for approximately 48 hours at room temperature. The precipitin bands were interpreted before and after staining with various protein stains (Appendix V).

2.7.2 Crossed immunoelectrophoresis

Two dimensional immunoelectrophoresis was used for the characterisation of the T. saginata metacestode antigens. The technique followed was essentially that described by Weeke (1973), with slight modifications as follows: The gel used consisted of 1% (W/v) of Litex agarose in sodium barbital calcium lactate buffer to which 1% Triton X-100 and 1% polyethylene glycol 6000 had been added (Appendix IV). The gel was 1.5 mm thick in the first dimension electrophoresis. The antigen well contained 15 ul of antigen solution. The first dimension electrophoresis was carried out for 60 minutes at 10V/cm. After completion of the first dimension electrophoresis, the agar strip was cut out and transferred to 50x50 mm glass plates for the second dimension electrophoresis. The second dimension electrophoresis was carried out using the same agarose composition, but mixed with

0.5ml of antiserum to make a final volume of 3mls. The electrophoresis was carried out at 2V/cm for 20 hours. The gel was pressed as described by Weeke (1973) and washed in 3% trisodium citrate, pH 8.2, for 24 hours, in order to remove non-precipitated proteins. After washing, the plates were rinsed in distilled water, pressed, dried and stained using stains shown in Appendix V.

2.7.3 Crossed immunoelectrophoresis with intermediate gel

This method was used to demonstrate the specificity of different antisera described in 2.6.4. The technique followed was that described by Axelsen (1973a). Briefly, 0.2 ml of specific antiserum or normal serum was mixed with the same agarose type used in crossed immunoelectrophoresis to make a final volume of 1 ml. This was poured to make the intermediate gel. The reference antiserum (0.5ml) was mixed with agarose to make a final volume of 2 mls which was poured onto the plate. The other procedures from this stage onwards were performed as already described in 2.7.2.

2.7.4 Tandem crossed immunoelectrophoresis

This method was used to compare the crossreacting antigens of T. saginata metacestodes and the various parasites.

The technique followed was essentially that described by Kroll (1973). The two antigen wells were separated by 7mm, center to center. The antigenic extract which was being compared was added to the most anodal wells, while the other

antigen well contained the reference antigen. First and second dimension electrophoresis were performed as described in 2.7.2.

RESULTS

3.1 IMMUNODIFFUSION TESTS

The results of immunodiffusion tests between rabbit anti-IMS and IMS and other parasites are presented in Tables 2 and 3. A positive sign (+) has been used to indicate the presence of a precipitin line, while a negative sign (-) indicates that no precipitin line was observed. Precipitin lines were labelled starting from the one nearest to the well containing the antiserum (Appendix III).

Rabbit anti-IMS serum showed five precipitin lines with the homologous antigen IMS. Four precipitin lines were observed with cyst fluid (F) and T. saginata whole worm extract (Table 3). The precipitin line labelled (C) has previously been defined as Antigen 11 in crossed immunoelectrophoresis. This antigen will, therefore, be referred to as Antigen 11 hence forth (Fig.1).

Antigen 11 is also present in the following parasite extracts: T. solium metacestode, T.hydatigena and its metacestodes, but is absent in some cestodes, such as Moniezia spp and T. crocutae (Table 2). This antigen is therefore present in only a few parasites. Precipitin line (E) was common to all cestodes examined in this study. Therefore, this precipitin line was tentatively considered as a cestode specific (Fig. 2). A specific antiserum was prepared from this precipitin band. Using crossed immunoelectrophoresis with intermediate gel, this precipitin line was found to be Antigen 4 in the reference pattern.

TABLE 2.

Table 2. (Cont'd)

T.solium metacestode	-
Avitellina spp	-
Spirometra spp	-
Stilesia hepatica	-
T.taeniaeformis stobilocercis	-
D.caninum	-
F.gigantica	-
Schistosoma bovis	-
Schistosoma mansoni	-
Paramphistomum spp	-
Paramphistomum spp from wildebeest	-
Cooperia spp	-
Oesophagostomum spp	-
Setaria digitatum	-
Setaria equinum	-
Trichostrongylus spp	-
Bunostomum spp	-
Ancylostomum caninum	-
Toxocara canis	-
Ascaris suum	-
Ascaridia gali	-
Haemonchus spp	-
Spirocerca lupi	-

Table 3. Immunodiffucion results showing precipitin lines common to *T. saginata* and its metacestode.

	Crossed immuno electrophoresis Antigen No.				
	Antigen 8	?	Antigen 11	Antigen 10	Antigen 4
Extract	A	B	C	D	E
T. saginata metacestode IMS	+	+	+	+	+
(F)	+	?	+	+	+
" (OM)	-	-	-	-	-
T. saginata whole worm	+	-	+	+	+
T. saginata eggs	+	-	-	+	+
T. saginata oncospheres	?	-	?	-	+

? = unidentified indistinct precipitin line

40d.

Precipitin band (D) was found to be widely distributed among other parasites. A specific antiserum was prepared against this line and was shown to correspond to Antigen 10 in the crossed immunoelectrophoresis reference pattern.

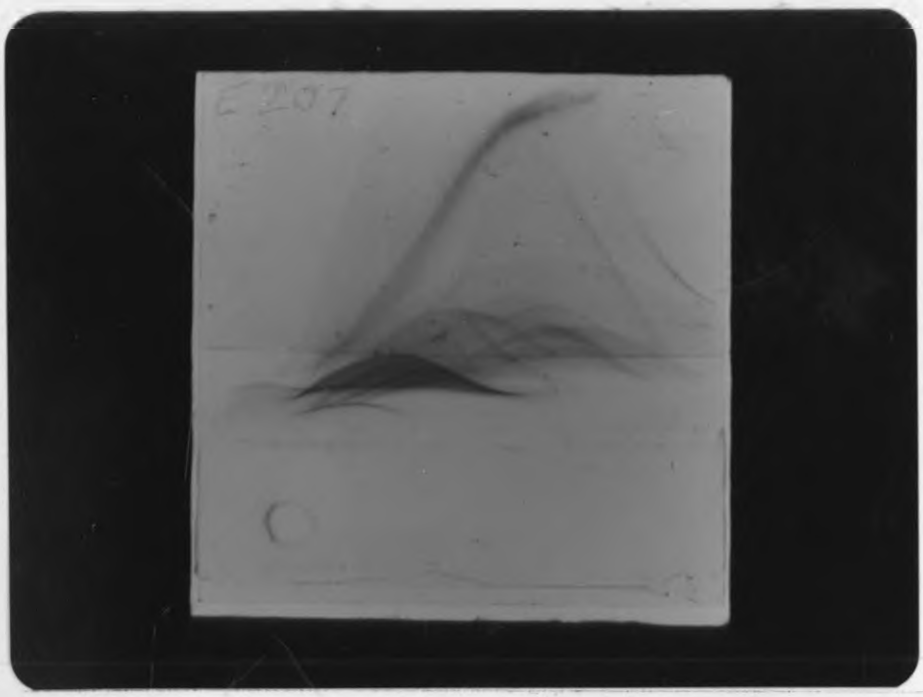
Precipitin band (A) was found to be restricted to T. saginata and its metacestode. This antigen was absent in all the other 30 spp of parasites examined. Therefore, this antigen was tentatively considered a species specific antigen (Fig.3). A specific antiserum was prepared against this antigen and it was identified as Antigen 8 in crossed immunoelectrophoresis.

Precipitin band (B) was found to be present in the T. saginata metacestode and the T. solium metacestode. No specific antiserum was made to this antigen and therefore, it was not identified in the reference pattern.

3.2 Reference pattern

The antiserum used to make a reference pattern was a pool of hyperimmune sera from 8 rabbits which had been immunized for a period of over 24 months (Appendx II).

The results of crossed immunoelectrophoresis are shown in Figure 1. The precipitin peaks were numbered starting with the most anodic component. Fifteen distinct precipitin arcs can be distinguished. This reference pattern was used for a detailed comparison of antigens shared between T. saginata metacestodes and other commonly occurring parasites. The reference pattern was also used to define the specificity of antisera produced in



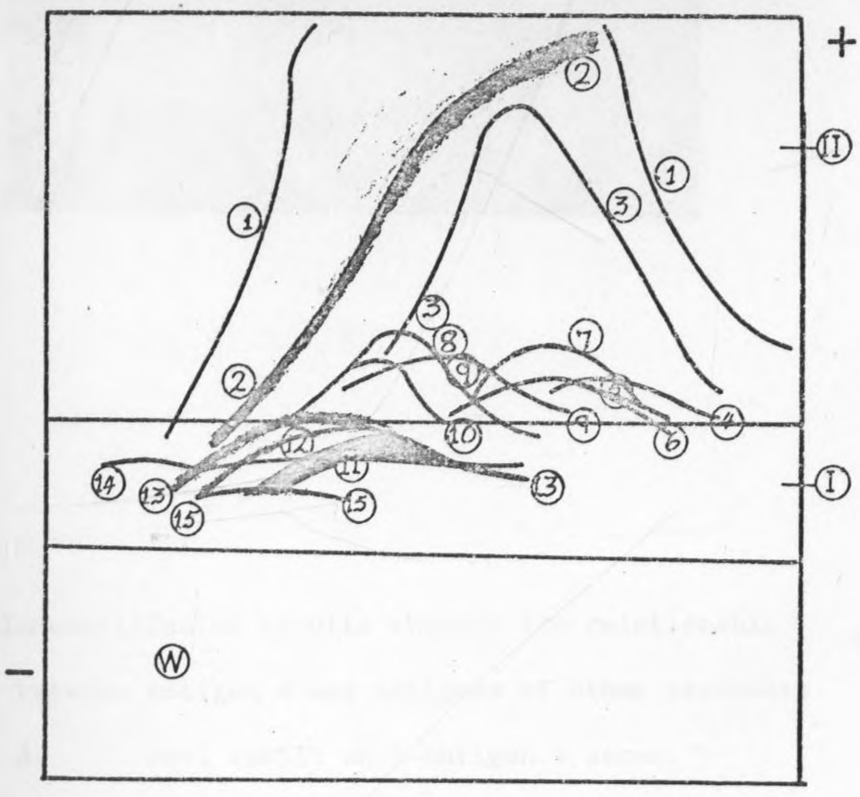


Fig. 1. Reference pattern for the antigen in constituents of T. saginata metacestodes as defined by Laurell crossed immunoelectrophoresis

(W) Antigen well: T. saginata metacestode extract (IMS)

(I) Intermediate gel: 0.2 ml normal rabbit serum in 1 ml gel

(II) Top gel: 0.5 ml reference antiserum; Rabbit anti-T. saginata metacestode serum in 2 ml gel.

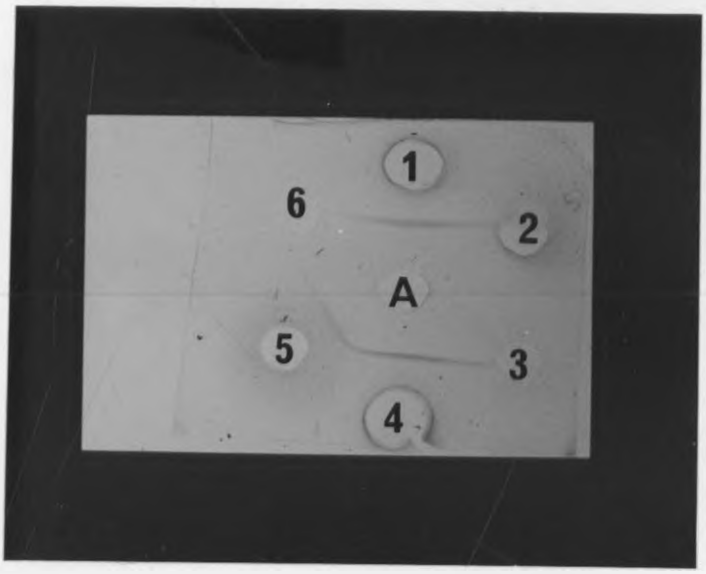


Fig. 2. Immunodiffusion results showing the relationship between Antigen 4 and antigens of other parasites.

A: Pool rabbit anti-Antigen 4 serum.

1. T. saginata metacestode extract (IMS)
2. Paramphistomum spp extract
3. Bunostomum spp extract
4. T. saginata metacestode extract (IMS)
5. T. hydatigena metacestode extract
6. Ascaris suum extract

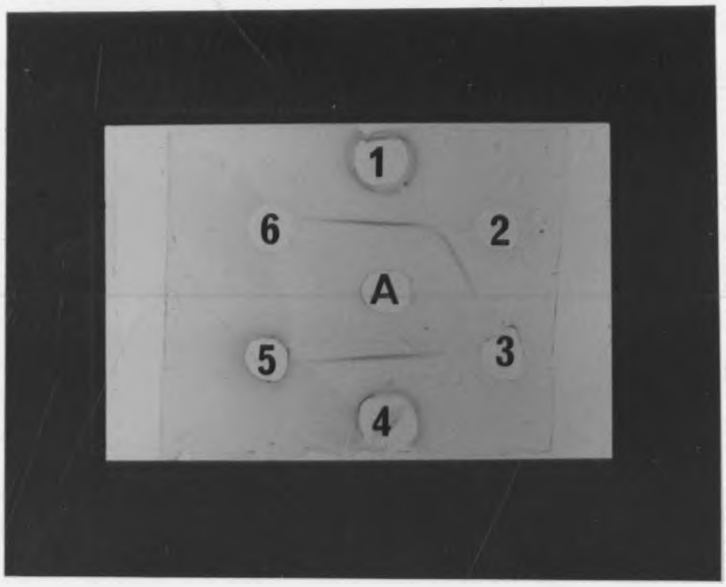


Fig. 3: Immunodiffusion results showing the relationship between Antigen 8 and antigen of other parasites

A. Pool rabbit anti-Antigen 8 serum

1. T. saginata metacestode extract (IMS)

2. T. saginata whole worm extract

3. T. hydatigena metacestode extract

4. T. saginata metacestode extract (IMS)

5. Ascaris suum extract

6. Paramphistomum spp extract

Table 4. Rf. values of 4 antigens relative to reference Antigen 8

Antigen	First dimension electrophoresis X Rf ± SD
7	1.6 ± 0.54
8	1.0
10	0.95 ± 0.05
11	0.85 ± 0.36
13	0.74 ± 0.23

experimental animals by immunization with precipitin lines obtained from immunodiffusion tests in agar.

Host serum components were not detected in this pattern when bovine serum was analysed, indicating that all the 15 precipitin arcs represented antigens of parasite origin.

One of the important prerequisite for a reference pattern is that of reproducibility. Evaluation of reproducibility was accomplished by selecting an internal reference point. For this purpose, Antigen 8 was selected since it showed the most distinct precipitin arc. It was consistently present in more than 100 runs of crossed immunoelectrophoresis, using different batches of IMS antigen preparations. The coordinates in both directions for the four other most distinct peaks, that is Antigens 7, 10, 11 and 13 were measured and expressed as Rf values relative to that of Antigen 8. The Rf value was expressed as the following ratio:

$$Rf = \frac{\text{distance of the peak from the point of origin (i.e. Antigen well)}}{\text{distance from origin to a reference point (i.e. Antigen 8).}}$$

Table 4 shows the values of this parameter for the four selected antigens measured in more than 100 experiments.

3.3 Antigens common to *T. saginata* and its metacestode.

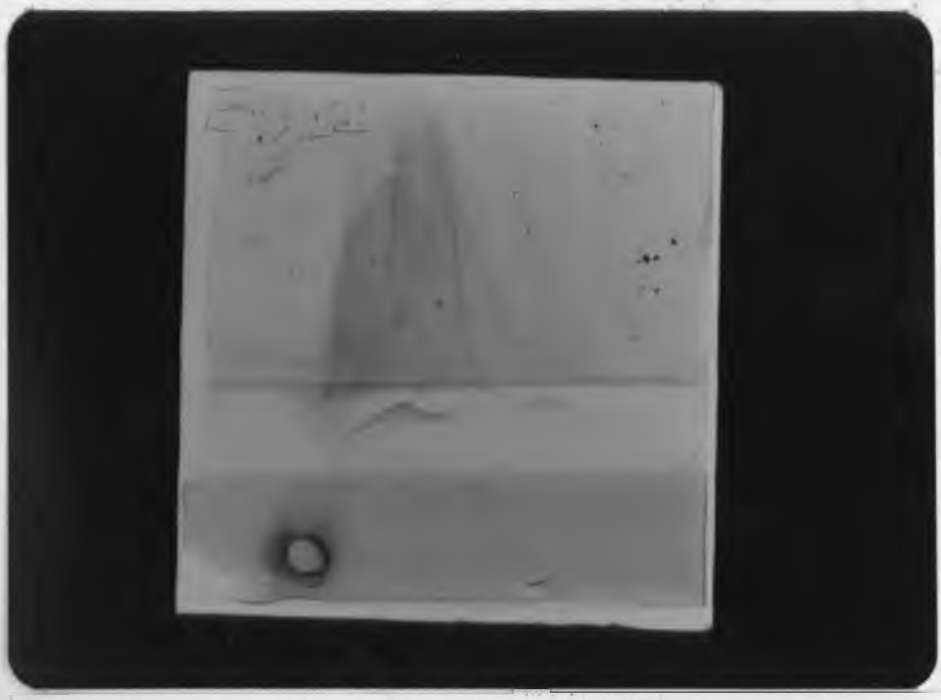
Table 5 shows the antigenic components which are shared between *T. saginata* and its metacestode. The cyst fluid (F) showed 7 common antigens (Fig.4) in contrast to the whole worm which showed 10 antigens (Fig.5). Three of these antigens were also present in *T. saginata* eggs, while only two antigens were demonstrated with *T. saginata* oncospheres.

The species-spefic antigen previously defined by immunodiffusion was identified as Antigen 8 (Fig.6). Other common antigens identified in *T. saginata* and cyst fluid (F) were Antigens 9, 10, 11 and 13. Four antigens, 2, 6, 14 and 15 were only found in IMS. These results were confirmed in tandem crossed immunoelectrophoresis analysis and in reciprocal crossed immunoelectroelophoresis analysis using antisera prepared against *T. saginata* whole worm extract (Fig.7) and antisera prepared against the cyst fluid (Fig.8).

Table 5. Antigens common to the whole worm, eggs, oncospheres and metacestode of T. saginata

Extracts	Antigen No. 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
T.saginata metacestode (IMS)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
T.saginata metacestode(F)	-	-	-	-	-	-	-	+	+	+	+	-	+	+	+
T.saginata metacestode(OM)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
T.saginata Whole Worm	+	-	+	+	+	-	+	+	+	+	+	-	+	-	-
T.saginata eggs	-	-	-	-	-	-	-	+	-	+	+	-	-	-	-
T. saginata oncospheres	-	-	-	+	-	-	-	?	-	+	-	-	-	-	-

? = unidentified indistinct precipitin line.



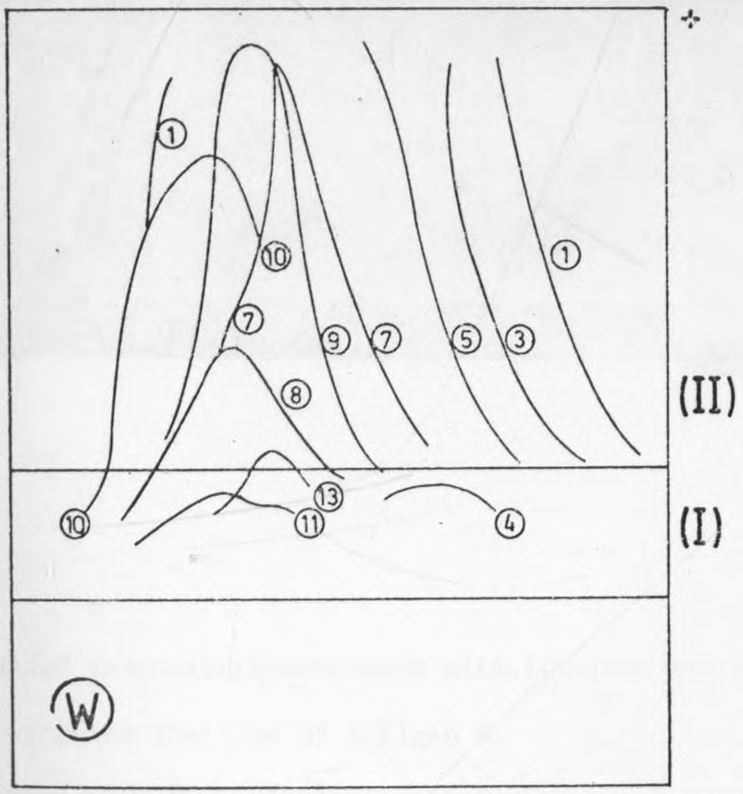


Fig.5: Laurell crossed immunoelectrophoresis showing the antigens shared between T. saginata and its metacestode.

(W) Antigen well: T. saginata extract

(I) Intermediate gel: 0.2 ml normal rabbit serum

(II) Top gel: 0.5 ml reference anti-T. saginata metacestode serum in 2 ml gel.

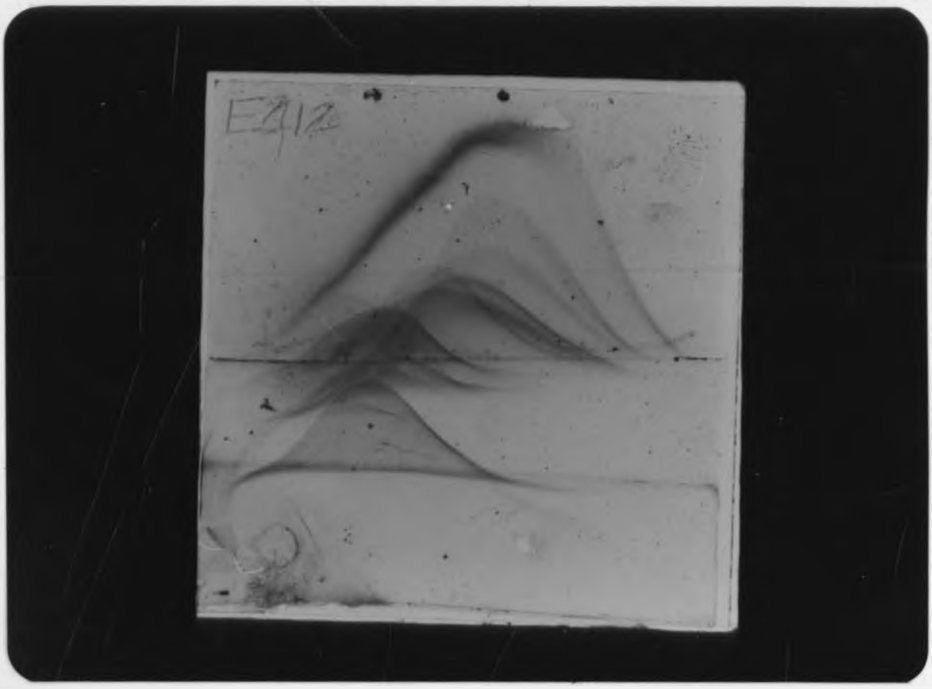
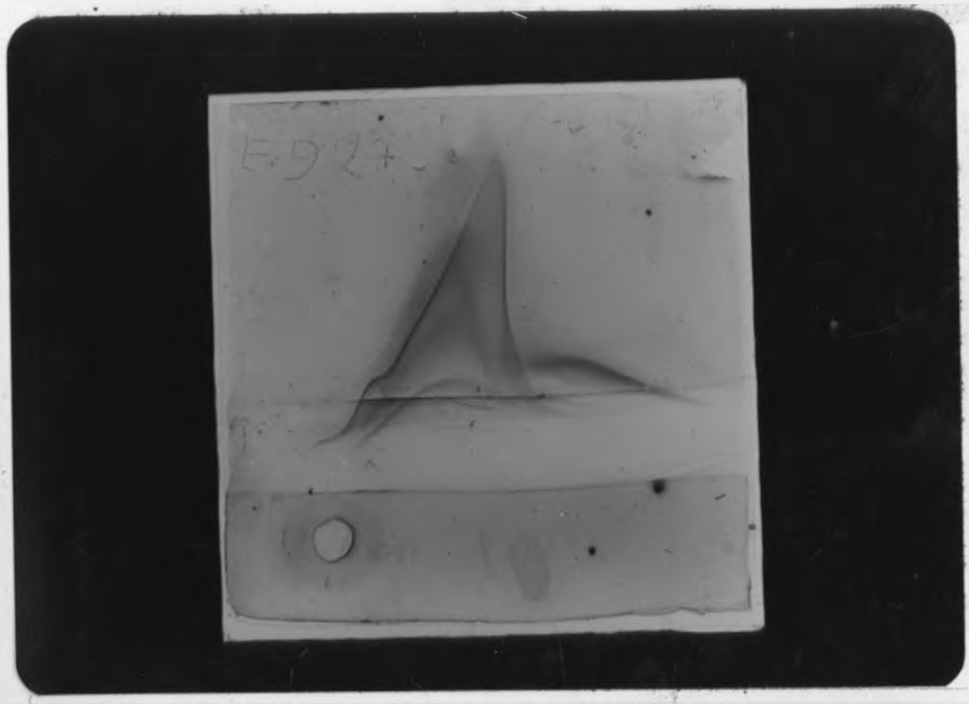


Fig. 6:Crossed immunoelectrophoresis with intermediate gel showing the position of Antigen 8.

(W) Antigen well T. saginata metacestode extract (IMS).

(I) Intermediate gel 0.2 ml of pooled specific antiserum to Antigen 8 in 1 ml gel.

(II) Top gel: 0.5 ml reference anti-T. saginata metacestode serum in 2 ml gel



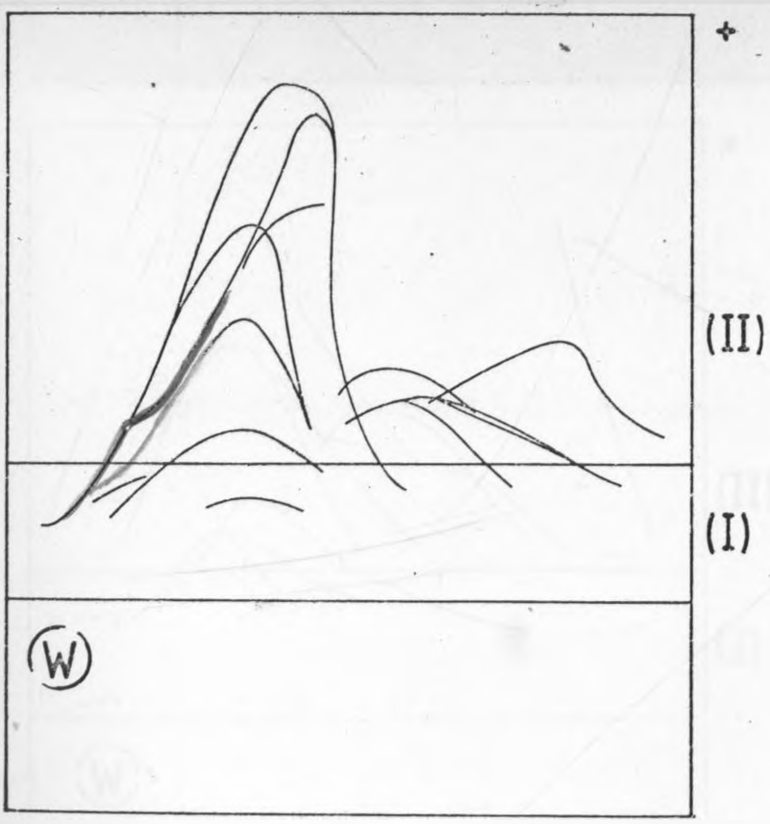
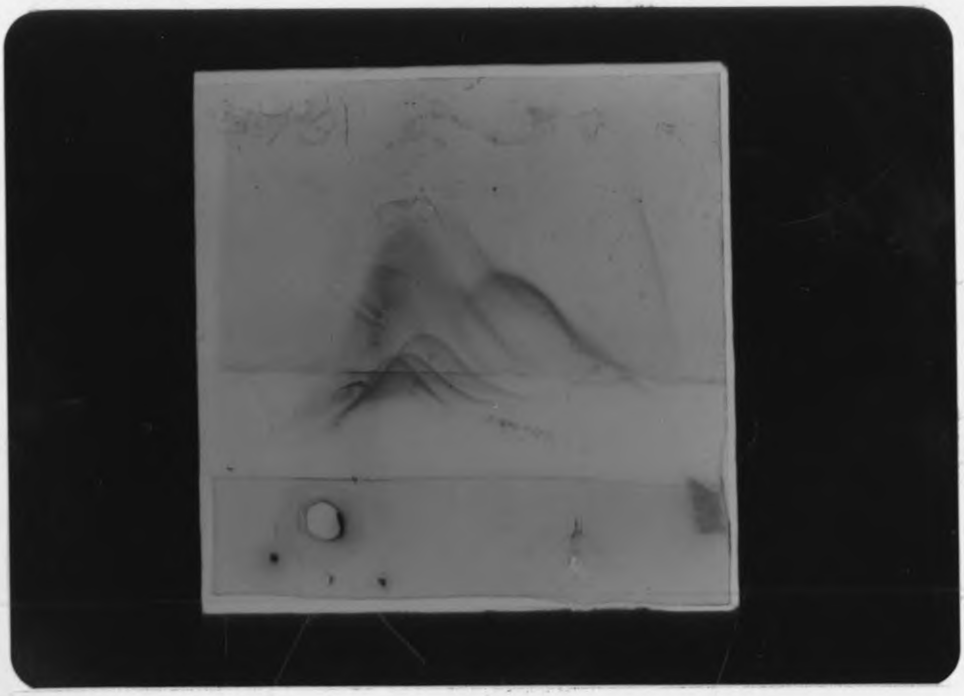


Fig. 7: A crossed immunoelectrophoresis pattern showing the presence of shared antigens between T. saginata and its metacestode.

(W) Antigen well. T. saginata metacestode extract

(I) Intermediate gel. 0.2 ml normal rabbit serum in 1 ml gel

(II) Top gel. 0.5 ml rabbit anti-T.saginata serum in 2 ml gel.



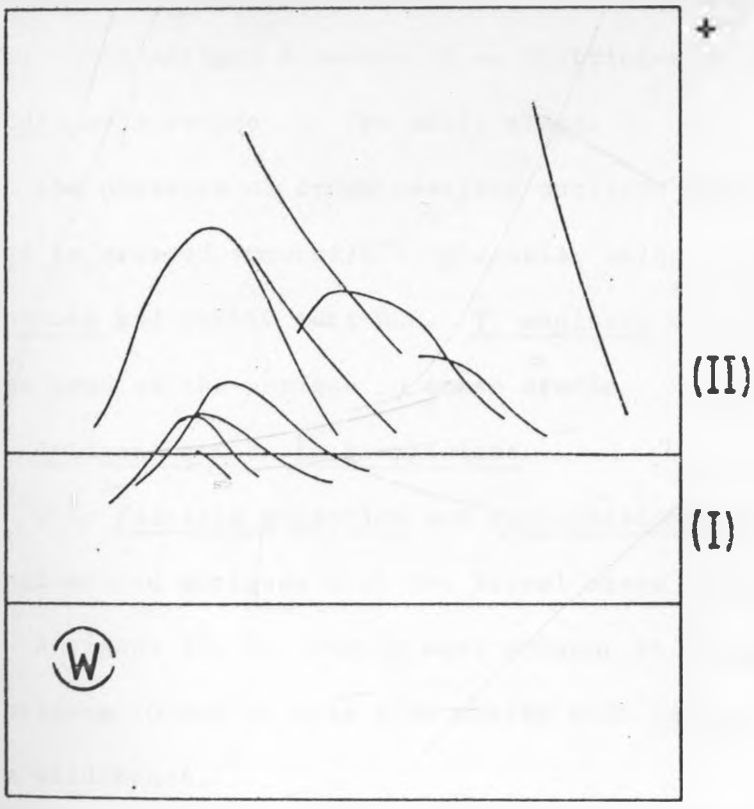


Fig. 8: A crossed immunoelectrophoresis pattern showing the presence of shared antigens between T. saginata metacestode extract (IMS) and cyst fluid (F).

(W) Antigen well: T. saginata metacestode extract (IMS)

(I) Intermediate gel): 0.2ml normal rabbit serum in 1 ml gel.

(II) Top gel: 0.5 ml rabbit anti-Inner fluid in 2 ml gel.

3.4 Antigens common to cestodes

Table 6 gives a summary of antigens detected in various cestode extracts using the reference rabbit anti-IMS serum. Antigen 4 appears to be cestode specific. Among all the cestodes examined, only Spirometra spp and Moniezia spp did not show the presence of this antigen. Antigen 4 was absent in the 17 non-cestode parasites examined.

There is a wide range of other antigens which are shared between the T. saginata metacestodes and other cestodes. Only Antigen 8 seemed to be restricted to the T. saginata metacestode and its adult stage.

The presence of cross-reacting antigens were also confirmed in crossed immunoelectrophoresis, using rabbit anti-Moniezia and rabbit anti-HCF. T. saginata metacestode (IMS) was used as the antigen in these studies.

3.5 Antigens common to trematodes:

Only Fasciola gigantica and Paramphistomum spp from wildebeest shared antigens with the larval stage of T. saginata.

Antigens 10, 14, and 15 were present in F. gigantica, while Antigens 10 and 14 were also shared with Paramphistomum spp from wildebeest.

The presence of shared antigens between T. saginata metacestodes and F. gigantica were confirmed in crossed immunoelectrophoresis using rabbit anti-Fasciola serum.

TABLE 6.

Table 6. Antigens common to cestodes

Extracts	Antigen No. 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
T.saginata metacestode (IMS)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
T.saginata metacestode Fluid (F)	-	-	-	-	-	-	-	+	+	+	+	-	+	+	+
T.saginata Whole worm	+	-	+	+	+	-	+	+	+	+	+	-	+	-	-
Moniezia sp.	+	-	-	?	-	+	-	-	-	+	-	-	+	-	-
T.hydatigena	+	+	+	-	-	-	-	+	+	+	+	-	+	-	-
T.hydatigena metacestode	+	-	+	+	-	-	-	-	+	+	+	+	+	-	-
T.crocutae	+	+	-	+	-	+	-	-	-	-	-	+	+	-	-
T.crocutae metacestode	+	+	-	+	-	+	+	-	+	+	-	+	+	-	-

T.crassiceps metacestode + + – +

Table 6. (Cont'd)

Hydatid cyst fluid	–	–	–	+	–	–	–	–	–	+	–	–	–	–	–
T.multiceps metaecestode	+	–	–	+	–	–	–	–	+	+	–	–	–	–	–
T.solium metacestode	–	–	–	+	–	–	–	–	–	+	+	+	+	–	–
Avitellina Spp	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–
Spirometra Spp	+	–	–	–	–	–	+	–	–	–	–	–	–	–	–
Stilesia hepatica	+	–	–	+	–	–	–	–	+	–	–	–	–	–	–
T.taeniaeformis strobilocercus	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–
D. caninum	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–

? = unidentified indistinct precipitin line

Table 7. Antigens of T. saginata metacestode that are shared with trematodes

Extract	Antigen No. 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
T.saginata metacestode	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
F. gigantica	-	-	-	?	-	-	-	-	-	+	-	-	-	+	+
Schistosoma bovis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Schistosoma mansoni	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Paramphistomum Spp	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Paramphistomum spp from wildebeest	-	-	-	-	-	-	-	-	-	+	-	-	-	+	-

? = unidentified indistinct precipitin line

44d.

3.6 Antigen common to nematodes:

All nematode species tested showed the presence of Antigen 10 (Table 8). This antigen, however, is not specific for this group of parasites since it has also been demonstrated in cestodes (Table 6) and trematodes (Table 7). This antigen, therefore, appears to be widely distributed among several unrelated parasites.

3.7. Synopsis of the antigenic relationship between T. saginata metacestodes and other parasites

All fifteen antigens of T. saginata metacestodes showed some degree of cross-reactions with cestodes, trematodes and nematodes commonly parasitizing domestic animals.

Antigen 4 was shown to be common among cestodes, while Antigen 10 appeared to be widely distributed among different unrelated parasites. These two antigens showed reaction of partial identity with T. saginata Fraction 10 produced by Dr. Geerts and his co workers (1981c).

Antigen 8 was shared only between T. saginata and its metacestode. This antigen was present in all stages of T. saginata.

Antigen 11 was present only in the T. solium metacestode and in T. hydatigena and its metacestode. This antigen showed a reaction of non-identity with Dr. Geerts' Fraction 10. In contrast, Antigen 13 showed cross reactions with the same parasites as Antigen 11, but was also present in T.crocutae metacestodes.

Table 8. Antigens Common to Nematodes

Extract	Antigen No. 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
T.saginata metacestodes	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Cooperia spp	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
Oesophagostomum spp	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
Setaria digitatum	-	-	-	-	-	-	-	-		+	-	-	-	-	-
Setaria equinum	-	-	-	-	-	-	-	-		+	-	-	-	-	
Trichostrongylus spp	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
Bunostomum spp	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
Ancylostoma canimum	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
Toxocara canis	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
Ascaridia galli	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
Ascaris suum	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
Haemonchus spp	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
Spirocerca lupi	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-

Antigens 4, 8, and 11 showed reactions of non-identity in immunodiffusion (Fig.9). This indicates that these are three distinct antigens of T. saginata metacestodes.

Fig. 9: Immunodiffusion results showing the relationship between Antigen 4, 8, and 11.

A. T. saginata metacestode extract (IMS)
1. Rabbit anti Antigen 8
2. Rabbit anti Antigen 4
3. Goat anti Antigen 11
4. Saline
5. Saline
6. Pool Goat anti Antigen 11.

DISCUSSION

4. DISCUSSION

Crossed immunoelectrophoresis is a superior method in comparison with the immunodiffusion techniques for the analysis of complex antigenic mixtures of infective agents. In this study, fifteen antigens of T. saginata metacestodes were defined using crossed immunoelectrophoresis as compared to only five revealed by immunodiffusion tests.

The reproducibility of the precipitin arc pattern obtained in crossed immunoelectrophoresis when antigen/antibody reactions were evaluated as coordinates relative to an internal reference point (Antigen 8) was excellent. Axelsen (1973b) used a similar approach which he designated as relative migration velocity, by mixing purified human albumin with an antiserum to Candida. Huppert et al. (1978) used an approach similar to the one followed in this study, to establish a reference pattern for the antigenic mosaic of coccidioidin and spherulin antigens from the dimorphic fungus Coccidioides immitis.

The excellent reproducability of the crossed immunoelectrophoresis technique indicated that the IMS/rabbit-anti IMS system can be recommended as a reliable standard reference pattern for the antigens of this parasite. Since the area under an antigen-antibody precipitate is related to the quantity of antigen and antibody present (Axelsen 1973a), it is possible to evaluate different batches of crude antigen preparations both in qualitative and quantitative

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terms. The method therefore lends itself as an excellent method for the standardization of antigens to be used in immunological tests.

The reference system is also a useful tool for monitoring successive steps during fractionation procedures aimed at the isolation of pure antigens.

The reference pattern has been used in the present study to compare antigens which are shared between T. saginata metacestodes and various commonly occurring parasites. Most antigens studied were shown to cross-react with other common parasites. This finding is similar to that reported by Biguet et al. (1965); Capron et al. (1968), and Geerts et al. (1979). A notable exception is Antigen 8 of T. saginata which was not detected in any of the 30 parasite species examined.

Geerts and his coworkers (1979) identified 22 precipitating components of T. saginata using immunoelectrophoretic analysis. They showed that only 7 antigens seemed to be of T. saginata origin. These authors also identified an antigen which they labelled Fraction 10. In the present study, Fraction 10 of Geerts et al. (1979) was shown to share antigenic determinants with Antigen 4 and with Antigen 10, both of which were found to be widely distributed among the parasites examined. Thus, these results are in agreement with the findings of Geerts et al. (1981c).

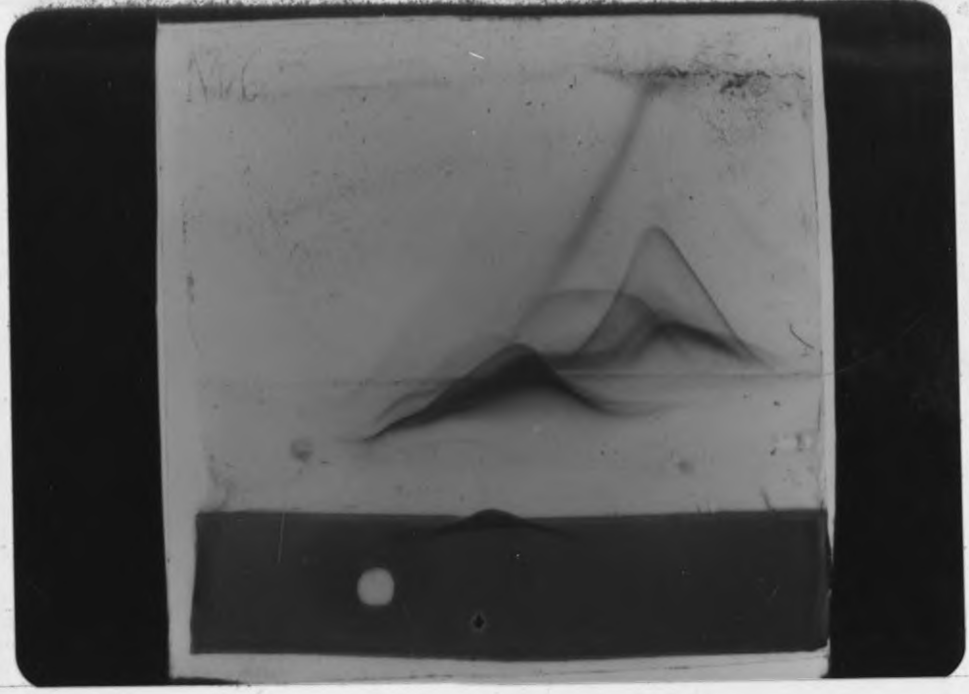
Geerts et al. (1981c) also attempted to isolate a species-specific antigenic fraction from T. saginata and concluded that this was an extremely difficult task. Through

the establishment of a reference pattern for the antigens of T. saginata and by examining 30 parasites using crossed immunoelectrophoresis, Antigen 8 was in the present study found to be specific for T. saginata. The production of a specific anti-Antigen 8 serum allowed the isolation and purification of this component using affinity chromatography or isoelectric focusing. This approach is the subject of the investigations described in Chapter II.

Absorptions of antisera were performed to remove antibodies to host serum components which are present as contaminants of many antigenic preparations (Valera-Diaz and Coltorti, 1972; Kamanga-Sollo et al., 1983). Damian (1962) discussed the possibility that parasites and host shared "eclipsed antigens". He defined "eclipsed antigen" as those antigenic determinants of the parasite which resemble antigenic determiants of the host to such an extent that they are not recognized as being foreign to the host. Numerous host antigens have been identified in parasites, the major one being blood group substances in hydatid cysts (Cameron and Stavley, 1957; Ben-Ismail et al., 1980). No attempts have been made in the present study to identify antigenic components related to host tissues.

Antigenic determinants shared between parasites may differ in their general chemical structures, but may show immunological cross reactivity because they share small structural units which may be composed of relatively few sugars or amino acids (Jenkin, 1963). It is, therefore, important

that only "functionally pure" antigens are defined and isolated from the mass of crude antigen preparations. These antigens should possess none or few epitopes shared with other parasites. Indeed, three such antigens of T. saginata metacestode have been identified. These are Antigens 4, 8 and 11. Their isolation and further characterisation are fully discussed in Chapter II (Fig. 10 and Fig. 11).



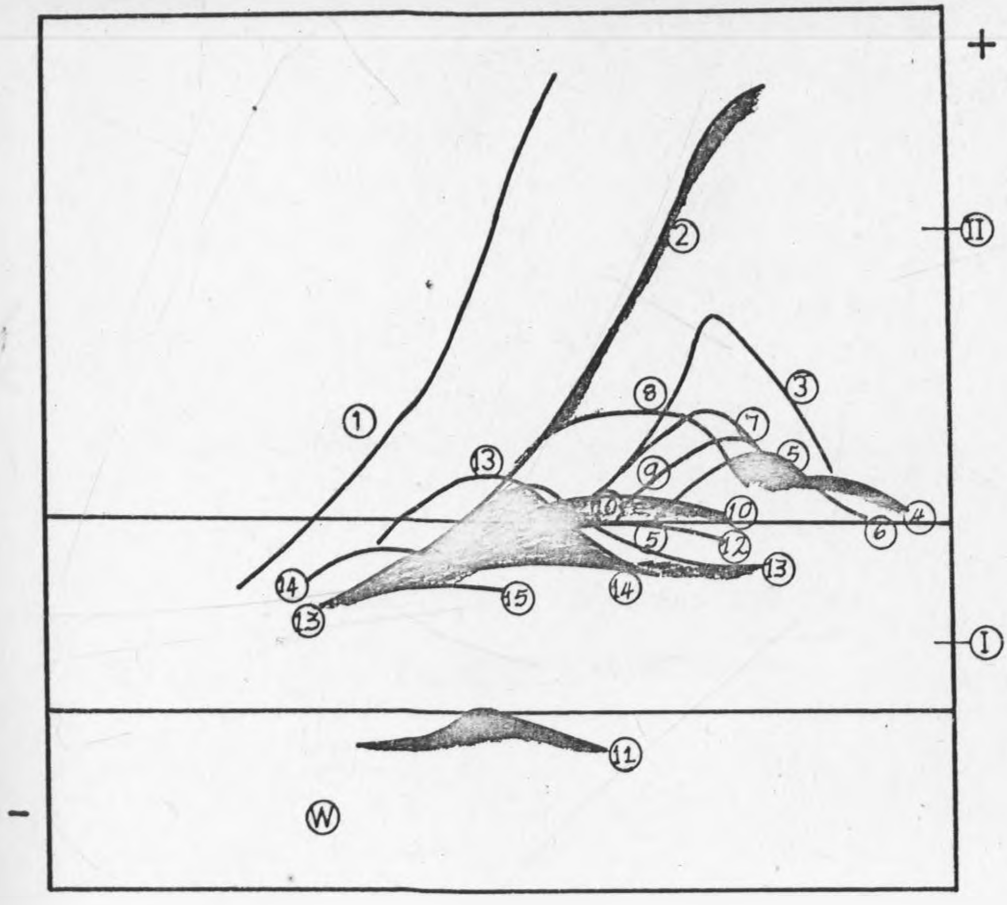


Fig. 11. Crossed immunoelectrophoresis pattern with intermediate gel showing the position of Antigen 11

(W). Antigen well: T. saginata metacestode extract (IMS).

(I). Intermediate gel: 0.2 ml of specific goat anti Antigen 11 serum in 1 ml gel.

(II). Top gel: 0.5 ml reference anti-T. saginata metacestode serum in 2 ml gel.

CHAPTER II

CHARACTERISATITON OF ANTIGENS FROM *T. SAGINATA* METACESTODES

1. INTRODUCTION AND REVIEW OF LITERATURE

Antigens of parasitic helminths can be studied for several fundamental or practical reasons. In the serology of parasitic diseases, there is a need for well characterised and specific antigens. This will enable the immunoparasitologist to standardize accurately and reproduce various tests employed in the immunodiagnosis of parasitic infections (Perry and Luffau, 1979). On the other hand, characterisation of antigens may enable a research worker to understand the role which antigens play in the pathology of disease, as well as the mechanisms which are used by the parasite to evade the defense mechanisms of the host (Perry and Luffau, 1979), and also provide an understanding of the immune responses and acquired immunity of the host to parasitic infection.

The development of immunodiagnostic procedures for helminthic diseases has essentially been based on attempts to select antigens possessing satisfactory specificity (Kent, 1963). Many modern physico-chemical fractionation techniques have been employed to isolate antigens for use in serodiagnostic tests. These have included the following methods: Alcohol extraction (Fairly and Williams, 1923; Minning and McFadzen, 1956), isoelectric precipitation (Melcher, 1943; Tarrant et al., 1965), ammonium sulfate precipitation (Urquhart et al., 1954), pyridine fractionation (Labzoffsky et al., 1956), chloroform gel fractionation (Fife and Kent, 1960), ethanol fractionation under controlled pH and

ionic strength (Reiber et al., 1961; Sleeman 1960, 1961), ion exchange and gel filtration chromatography (Kent, 1963; D'Antonio et al., 1966; Ohira, 1967; Morris et al., 1968; Khan and Meerovitch, 1970; Grossklaus and Walther, 1970; Gathuma and Waiyaki, 1980), immunoelectrophoresis (Sawada et al., 1964), and affinity chromatography (Bout et al., 1974; Suzuki et al., 1975).

Chemical fractionation does not necessarily lead to "pure" antigens (Tanner and Gregory, 1961). For instance Melcher's acid-soluble extract of Trichinella larvae (Melcher, 1943) was still found to be a complex antigen which was similar to an antigenic preparation obtained by extraction of Trichinella larvae with borate buffer, pH 8 (Tanner and Gregory, 1961). An acid soluble protein fraction of cestode antigen prepared by Zapart et al. (1974) was found to be a complex mixture of polysaccharides and proteins.

Gel filtration of crude antigens using Sephadex G-200 and Sephadex G-75, provides a simple separation technique for obtaining antigens of different molecular weights and has been widely used for the preparation of parasite antigens (Hillyer and Santiago de Weil, 1977; Gathuma and Waiyaki, 1980, 1981; Belozorov and Gudonavitchus, 1982). Gathuma and Waiyaki, (1980) used the method to obtain partially purified antigens from mature proglottids of T. saginata. They obtained a fraction which was labelled F1 which was shown to be antigenically reactive.

In the present study, gel chromatography was used as a preparative stage in the isolation of crude antigens from T. saginata metacestode. The specific antigens were then isolated by affinity chromatography using the immunoglobulin fraction of specific antisera coupled to cyanogen bromide activated Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden). The antigens were further characterised using isoelectric focusing and the molecular weights of the isolated antigens determined by polyacrylamide gel electrophoresis in the presence of the detergent sodium dodecyl sulphate (SDS-PAGE).

The specificity and the purity of the eluted antigens was confirmed by immunodiffusion tests, crossed immunoelectrophoresis and enzyme immunoassays using antisera specific for these antigens, as well as polyvalent antisera.

MATERIALS AND METHODS

2.1 GEL FILTRATION CHROMATOGRAPHY

Gel filtration was carried out in a Pharmacia K26/100 column (Pharmacia Fine Chemicals, Uppsala, Sweden). Pharmacia Sephadex G-200 was prepared according to the manufacturer's recommendations. The buffer used for elution consisted of the following: 0.15M potassium phosphate buffer with 0.1M sodium chloride and 0.02% sodium azide as a preservative.

The column dimensions were 98x2.6 cm. The verification of satisfactory column packing and determination of void volume were accomplished with blue dextran 2000.

Sixty milligrams of lyophilized T. saginata metacestode (IMS) homogenate was dissolved in 3 mls of 0.15M potassium phosphate buffer containing 1M sodium chloride and 0.2% Nonidet-P40. The antigen solution was then centrifuged at 3000xg for 15 minutes in a refrigerated centrifuge (Minifuge 2, Heraus Christ, West Germany) and the supernant filtered through 0.45u (Millipore filter, Molshen, France). Three mls of clear filtrate was applied to the column. The column flow rate was adjusted to 18 ml/hr and 1.5 ml fractions were collected.

The protein contents of eluates from the column were determined using the Lowry method (Lowry et al., 1951), while the sandwich enzyme immunoassays were used to determine the presence of specific antigen in the fractions (see section 2.5).

2.2 Affinity chromatography

Three affinity chromatography columns were prepared by coupling IgG fractions from antisera possessing specificity for Antigens 4, 8, and 11. IgG fractions were prepared according to the method described by Fey et al. (1976) using DEAE-cellulose chromatography (Cellex D, BIORAD Laboratories, Richmond, California, U.S.A), 0.02M phosphate buffer, pH 8.0, as eluting buffer in a one step procedure.

The IgG fractions of various antisera were coupled to CNBr-Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) following the manufacturer's recommendation. Each column was first washed with 3.5M potassium thiocyanate in 0.15M phosphate buffer, pH 7.4, equilibrated with 0.1M borate buffer pH 8.0, and washed several times with saline. Antigens partially purified by filtration through Sephadex G-200 columns were recirculated through each affinity chromatography column. Elution of antigens was performed using 3.5M potassium thiocyanate in 0.15M phosphate buffer pH 7.4. The eluted antigen was desalted using Sephadex G-75 columns equilibrated with PBS and concentrated to the original volume using "DIAFLO PM10" ultrafilter (Amicon, Massachusetts, U.S.A.)

The antigens were analysed for specificity and purity by immunodiffusion, crossed immunoelectrophoresis and enzyme immunoassay. Their molecular weights were estimated using SDS-PAGE and their pI determined by isoelectric focusing (see Sections 2.5 and 2.6).

2.3 Immunodiffusion and crossed immunoelectrophoresis techniques

Ouchterlony's double diffusion test and Laurell's crossed immunoelectrophoresis as modified by Clarke and Freeman (1967) were performed as described by Weeke (1973). The buffers used were the same as those described in Chapter I.

2.4 Enzyme immunoassay

Two types of non-competitive heterogeneous enzyme immunoassays (EIA) were used to establish the specificity of antigens obtained by affinity chromatography.

The first assay involved the coating of Falcon microtitre plates (Dickinson and Co, Oxnard, California, U.S.A.) with antigen eluted from the immunosorbent columns, followed by enzyme-labelled specific antibody conjugate. In the second type of EIA, the plates were coated first with specific antibody, followed by eluates from affinity columns and enzyme-labelled specific antibody conjugate (sandwich enzyme immunoassay). The free enzyme-labelled antibody conjugate was separated from enzyme labelled antigen-antibody complex by washing the plate with PBS-Tween.

2.4.1 Conjugation of enzyme label to antibodies

Glucose oxidase (EC 1.1.3.4) type VII (Sigma St Louis, Mo, USA) was coupled to IgG fractions of various antisera using the heterobifunctional reagent N-succinimidyl-3-

(2-pyridyldithio) propionate (SPDP) (Pharmacia Fine Chemicals, Uppsala, Sweden) following the method described by Ishikawa et al. (1983).

2.4.2 The enzyme immunoassay procedure

In the non-competitive assay, the antigens eluted from the affinity column were coated to microtitre plates using 0.01M carbonate buffer, while in the sandwich assay the antiserum was diluted 1/1000 in 0.15M PBS coating buffer (Appendix IX). The antigens were adjusted to a concentration between 25 ug/ml and 100 ug/ml, while the crude saline extract from T. saginata metacestode (IMS) was diluted to a protein concentration between 20 ug/ml and 40 ug/ml, as determined by the method of Lowry et al. (1951).

The conjugates were diluted from 1/25 serially up to 1/3200 in a conjugate diluent (Appendix IX).

The enzyme immunoassays were performed basically as described by Voller et al. (1976) and Lindqvist et al. (1982). Briefly, the following steps were performed. The Falcon microtitre plate wells were coated with 100 ul of an appropriate dilution of antigen or antiserum. The plate was incubated overnight at room temperature in a humid box. The plate was then washed 5 times in PBS-Tween (Appendix IX). In the non-competitive antigen assay, 100 ul of the glucose oxidase conjugate was then added and the plate incubated at 37°C for 1 hour, while in the sandwich assay, the plate coated with appropriate antiserum was washed five times with

PBS-Tween solution, 100 ul of antigen dilutions added and the plate incubated for 1 hr at 37°C. The plate was then washed five times with PBS-Tween solution before the addition of 100 ul of the glucose oxidase conjugate. The final step involved the washing of the plate 5 times using PBS-Tween solution and the addition of 100 ul of substrate solution per well (Appendix IX). The optical density (OD) was read after 1 hr using Dynatech's "Minireader MR 590" (Dynatech Microtitre Systems) equipped with a 410 nm interference filter. All the tests were run in duplicate.

2.5 SDS-PAGE analysis

The method described by Weber and Osborn (1969) was used for SDS-PAGE analysis. Five percent acrylamide solution was used in all the experiments (Appendix VI).

2.6 Isoelectric focusing

Isoelectric focusing was performed in the 110 ml column (LKB 8100-1) according to the manufacturer's recommendations (LKB, Bromma, Sweden). LKB-Ampholine carrier ampholytes with a pH range of 3.5 to 10 were used to create the pH gradient (Appendix VII).

RESULTS

3. RESULTS

Elution profiles of antigens from Sephadex G-200 column are shown in Figure 12. It was difficult to separate the antigens completely with this method. Nevertheless, the antigens showed three definite peaks. The tubes containing peaks of several runs of the same antigens were therefore pooled. These were concentrated by ultrafiltration and specific antigens further purified by affinity chromatography.

Three specific antigens were eluted from immunoaffinity columns. These antigens showed reaction of non-identity in immunodiffusion analysis using polyspecific antiserum to T. saginata metacestode (Fig. 13) and with specific antisera raised against these antigens. They were confirmed to be Antigens 4, 8, and 11. Figure 14 (a;b;c;) shows the results of crossed immunoelectrophoresis analyses for these antigens. The enzyme immunoassay analyses using specific enzyme-labelled antiserum conjugates are shown in Figures 15, 16 and 17. In general, the sandwich assays showed higher OD readings than the non-competitive direct enzyme immunoassay.

Antigen 11 isolated from T. hydatigena metacestode had previously been found to have a molecular weight between 63kd and 78kd. The current results of analysis of this antigen isolated from T. saginata metacestode shows this antigen to have a molecular weight of 68 kd (Fig. 18).

Analysis of Antigen 8 on SDS-PAGE showed it to be a high molecular weight protein of 260 kd which could easily be reduced to three components with molecular weights of 110 kd, 72 kd and 61 kd (Figs. 18, 19 and 20). This antigen had a pI between 6.95 and 7.30, while Antigen 11 had a pI range between 5.62 and 6.56. Antigen 4 had a pI range of 5.21-5.86 and a molecular weight of 63 kd.

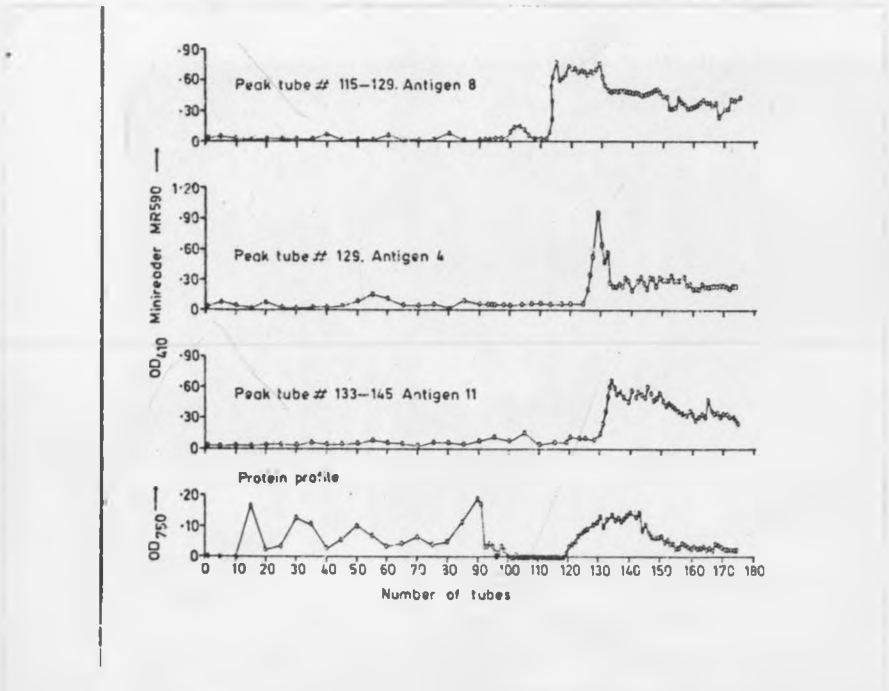


Fig. 12: Elution profile from Sephadex G-200 column of an extract from T. saginata metacestode (IMS).

Column: Pharmacia column K26/100 with flow adaptors.

Bed dimension: Length 98 cm, Diameter 2.6 c m.

Eluent: 0.15M potassium phosphate pH 7.5 with 0.1M sodium chloride, 0.2% Non idet P40 and 0.02% sodium azide.

Flow rate: 18.0 ml/hour

Void volume: 163.3 ml

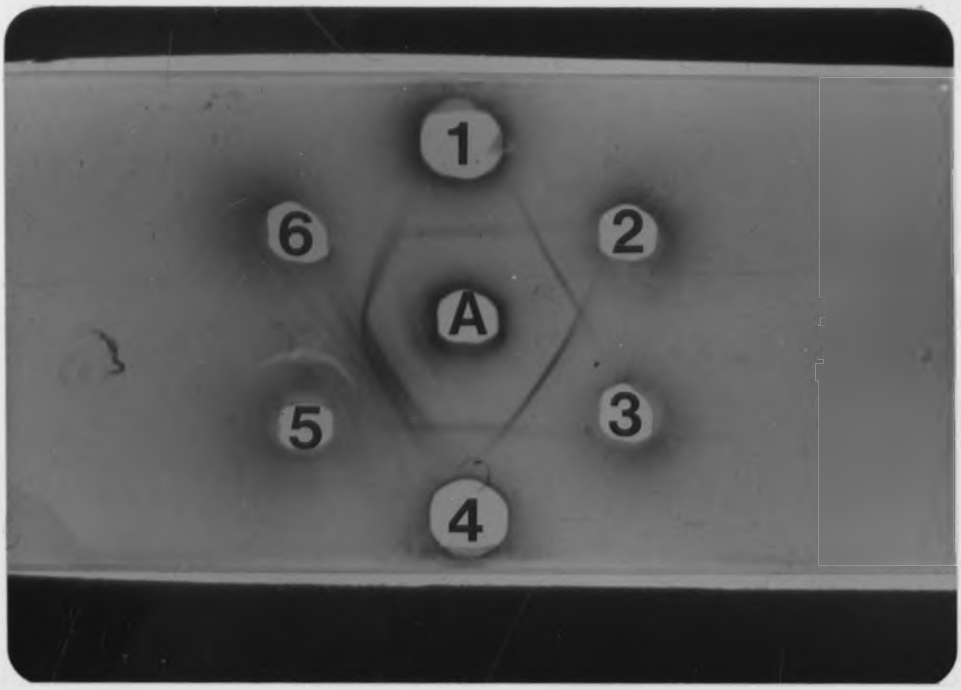


Fig.13: Immunodiffusion results showing the relationship between Antigens 4, 8, and 11, purified by affinity chromatography.

A. Reference anti-serum to T. saginata metacestode (Anti-IMS-serum).

1. Antigen 4.
2. Antigen 8.
3. Antigen 11.
4. Antigen 8.
5. T. saginata metacestode extract (IMS).
6. Antigen 11.

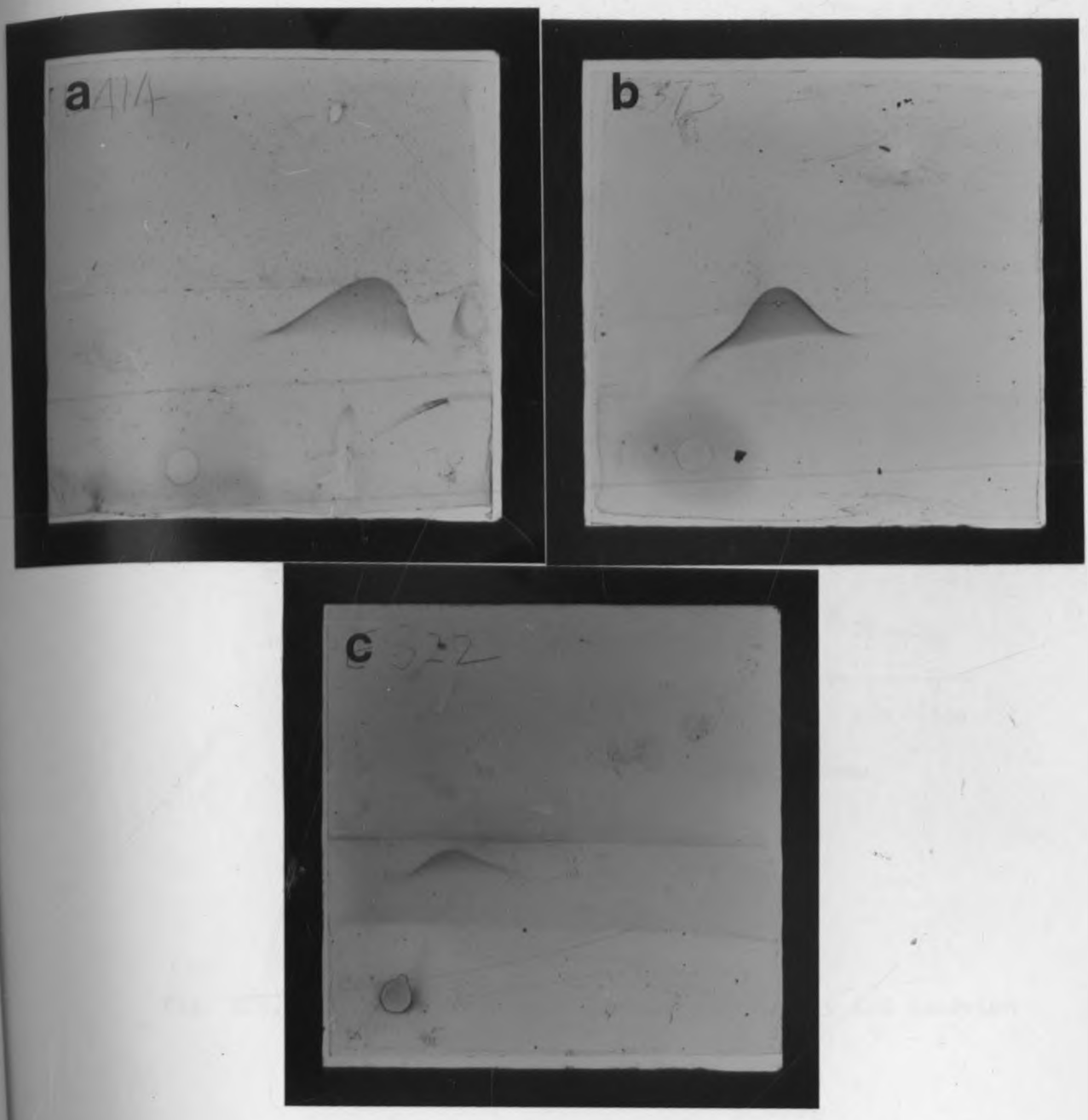


Fig. 14: Crossed immunoelectrophoresis showing the specificity of Antigens 4, 8, and 11.

14a. Antigen 4
14b. Antigen 8
14c Antigen 11.

(W) Antigen well. Specific Antigen.
(II. Top gel: Reference anti T. saginata metacestode serum 0.5 ml in 2 ml gel.

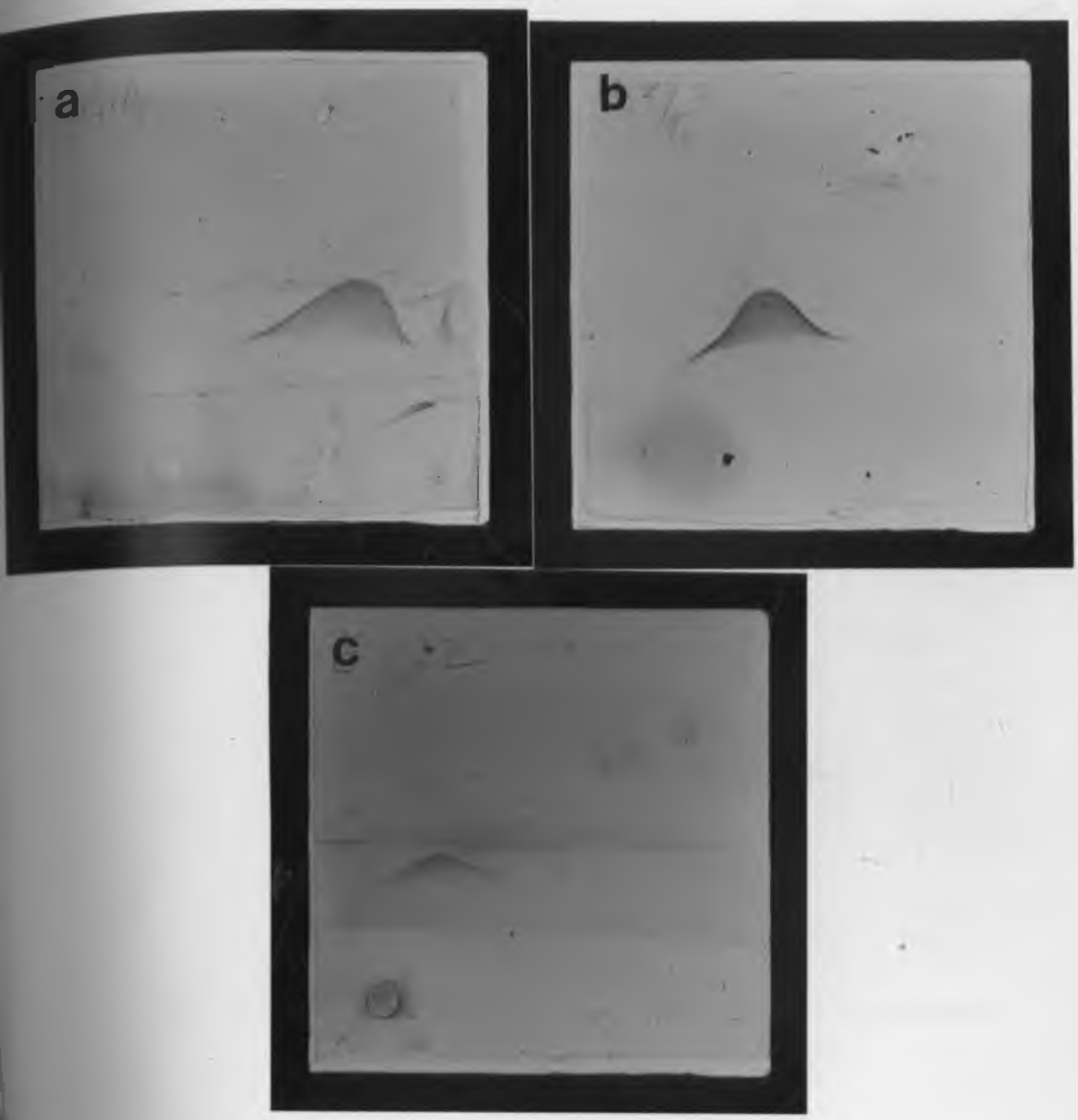


Fig. 14: Crossed immunoelectrophoresis showing the specificity of Antigens 4, 8, and 11.

14a. Antigen 4
14b. Antigen 8
14c Antigen 11.

(W) Antigen well. Specific Antigen.
(II. Top gel: Reference anti T. saginata metacestode serum 0.5 ml in 2 ml gel.

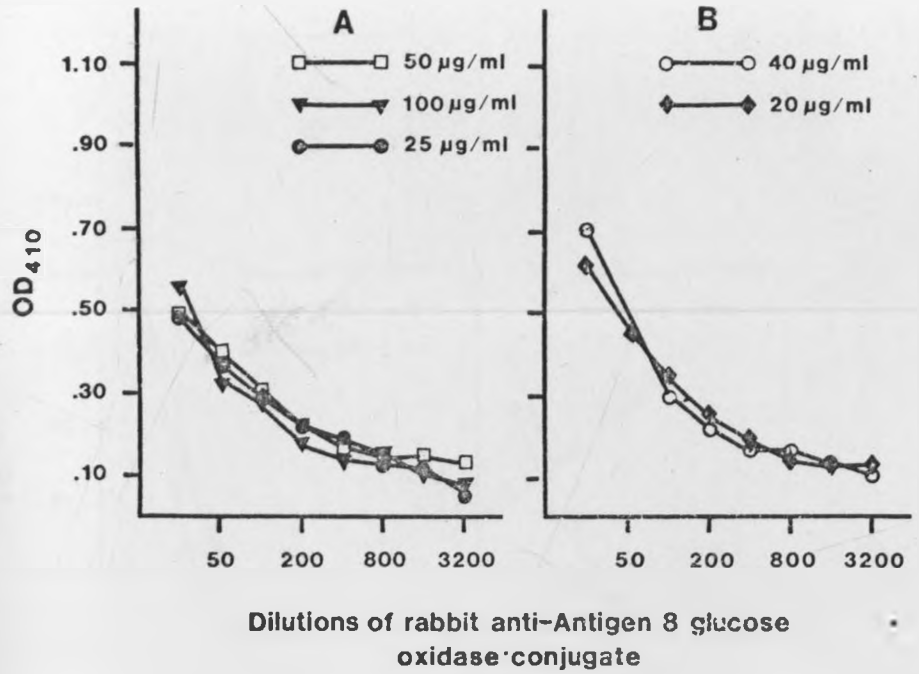


Fig. 15a: Comparison of direct enzyme immunoassay and sandwich enzyme immunoassay using Antigen 8.

Direct enzyme immunoassay using Antigen 8 purified by affinity chromatography (A); and crude saline extract of T. saginata metacestode (IMS) (B). Using glucose oxidase conjugate of rabbit anti-Antigen 8.

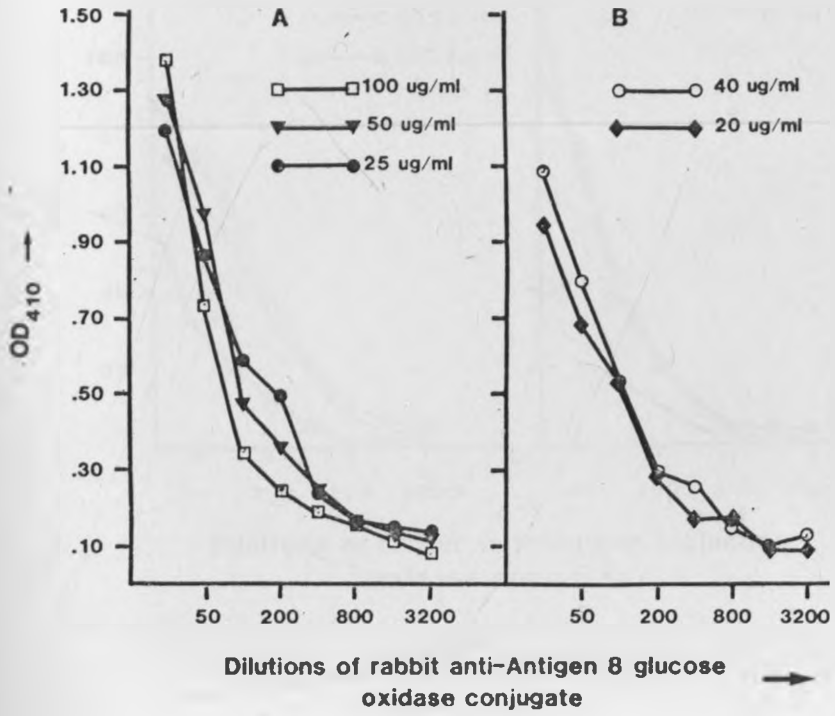


Fig. 15b Sandwich enzyme immunoassay using Antigen 8 purified by affinity chromatography (A); crude saline extract of T. saginata, metacestode (IMS) (B); and glucose oxidase conjugate of rabbit anti-Antigen 8 serum.

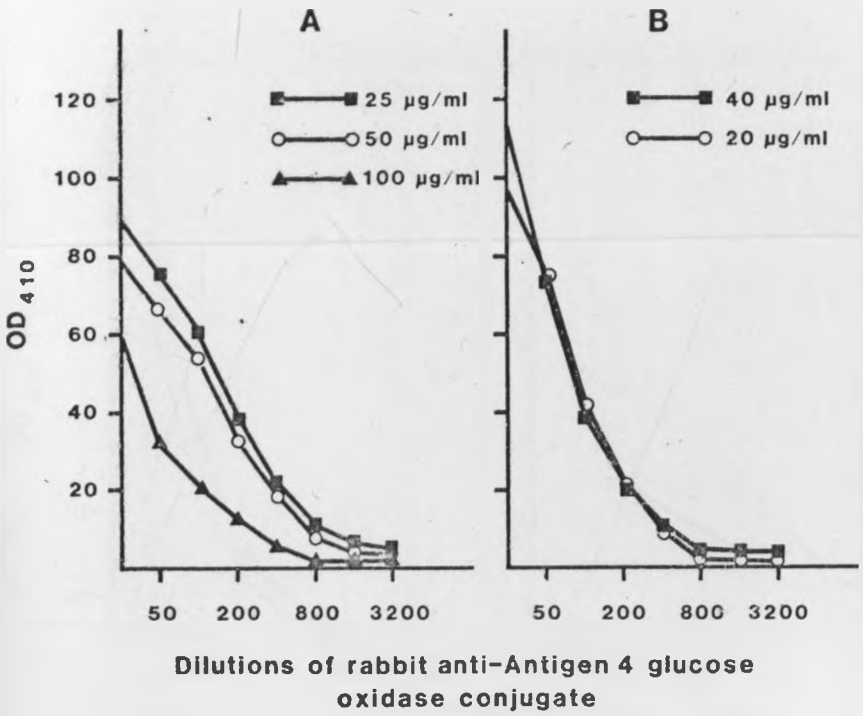


Fig. 16: Titration of Antigen 4 using glucose oxidase conjugate of rabbit anti-Antigen 4 serum in sandwich enzyme immunoassay.

A. Antigen 4 isolated by immunoaffinity chromatography.

B. Crude saline extract of T. saginata metacestode (IMS).

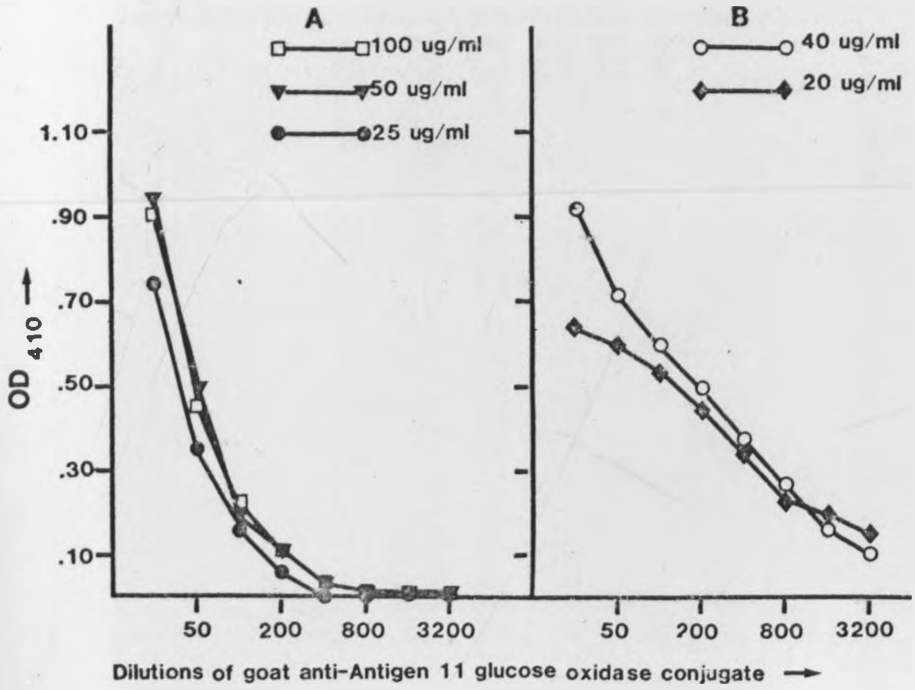


Fig. 17: Titration of Antigen 11 using glucose oxidase conjugate of goat anti-Antigen 11 in sandwich enzyme immunoassay.

A. Antigen 11 isolated by immunoaffinity chromatography.

B. Crude saline extract of T. saginata metacestode (IMS).

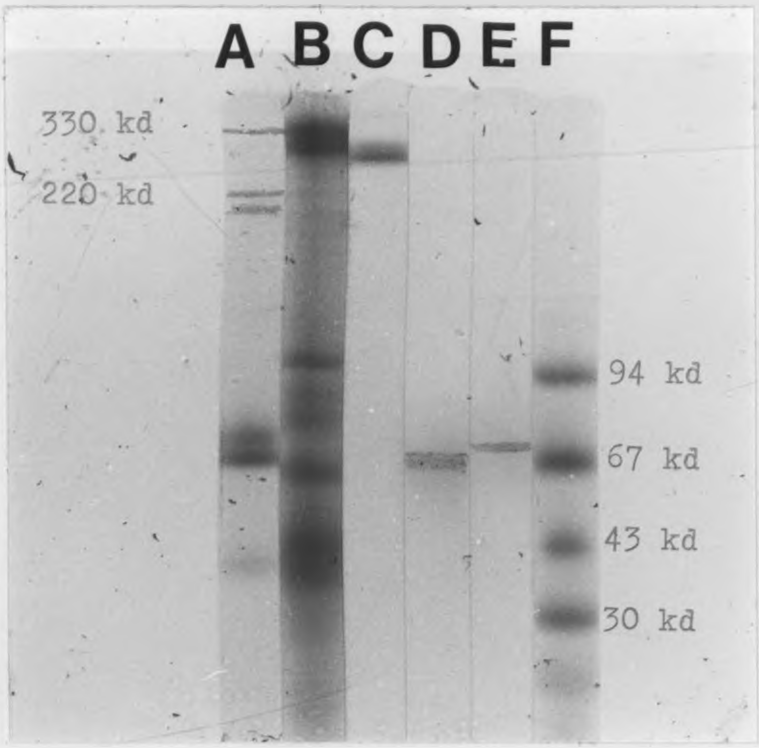


Fig. 18: SDS-PAGE analysis of Antigens 4, 8 and 11.

A. High molecular weight standard *
B. Crude saline extract of T. saginata metacestode
C. Antigen 8 purified by affinity chromatography
D. Antigen 4 " " "
E. Antigen 11 " " "
F. Low molecular weight standard *.

*Calibration kits for molecular weight determination using electrophoresis (Pharmacia Fine Chemicals, Uppsala, Sweden).

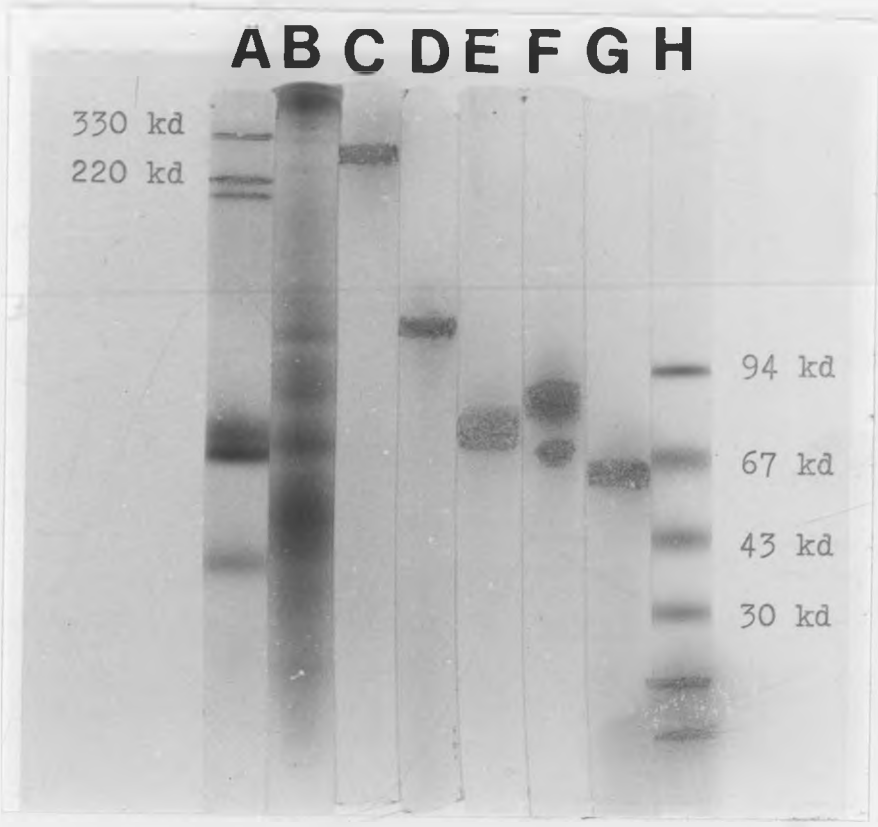


Fig. 19: SDS-PAGE analysis of Antigen 8.

A. High molecular weight standard*
B. Crude saline extract of T. saginata metacestode
C. Antigen 8 purified by affinity chromatography
D. Antigen 8 obtained after isoelectric focusing at pI 6.95.
E. Antigen 8 after reduction with mercaptoethanol (C).
F. Antigen 8 obtained after isoelectric focusing, and reducing with mecarptoethanol (D).
G. Antigen 8 obtained after isoelectric focusing at pI 5.70.
H. Low molecular weight*.

*Calibration kits for molecular weight determination using electrophoresis (Pharmacia Fine Chemicals, Uppsala, Sweden).

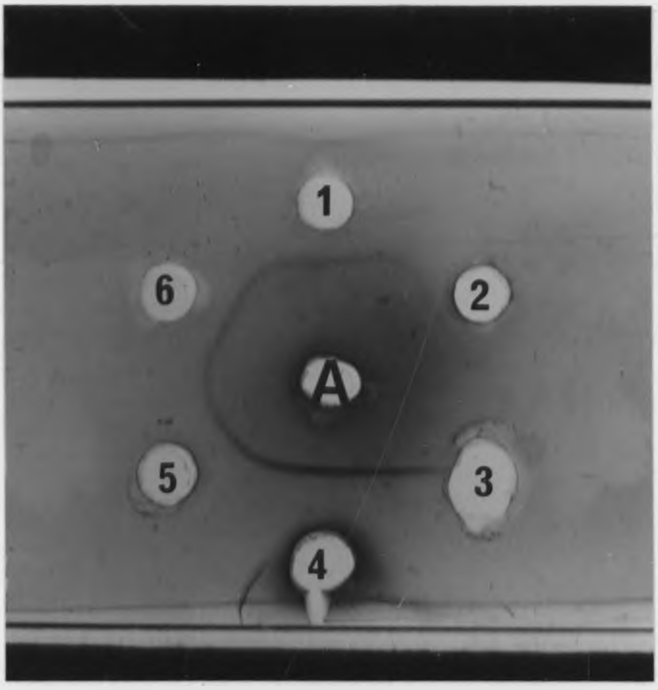


Fig. 20: Immunodiffusion results showing the relationship between the components of Antigen 8.

A. Rabbit anti-Antigen 8 serum

1. Antigen 8 purified by affinity chromatography (M.W. 260 kd)

2. Antigen 8 obtained after isoelectric focusing pI 6.95 (M.W.110 kd)

3. Antigen 11 purified by affinity chromatography

4. Crude saline extract T. saginata metacestode IMS

5. Antigen 8 obtained after isoelectric focusing pI 6.45 (MW.72 kd)

6. Antigen 8 obtained after isoelectric focusing pI 5.70 (M.W.61 kd)

DISCUSSION

4. DISCUSSION

Antigens 4, 8 and 11 were difficult to isolate using Sephadex G-200 exclusion chromatography since their molecular weights are nearly the same. Other types of exclusion gels such as Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) may have produced better results. This type of gel would probably have separated Antigen 8 from other low molecular weight antigens. However, Sephadex G-200 filtration was a useful procedure as a preparatory stage in the isolation of antigens by affinity chromatography.

Harrison and Sewell (1980) partially purified a saline extract of T. saginata proglottides by gel filtration chromatography on Sephadex G-200. They obtained two distinct fractions. The first fraction was of high molecular weight, while the second fraction had a molecular weight of approximately 100 kd. Gathuma and Waiyaki (1980) also isolated an antigenic fraction which they labelled F1. This antigen may be similar to the antigen of high molecular weight of Harrison and Sewell (1980) since they both showed a high level of activity in the indirect haemagglutination test.

Bolshakova et al. (1975) isolated antigens from T. saginata cysticerci using Sephadex G-75 and Sephadex G-200. However, it is doubtful that these workers obtained specific antigens since most of the specific antigens have been shown to have a close range of molecular weights.

4. DISCUSSION

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Antigen 4 occurs in a variety of cestode extracts (Chapter I) and elutes close to other antigens, such as Antigens 8 and 11, in Sephadex G-200 exlusion chromatography. It is, therefore, likely that Antigen 4 will contaminate these antigens in such a purification procedure. However, using immunoaffinity columns the three antigens can be separated specifically.

Several authors have used affinity chromatography for the purification of parasite antigens (Hillyer and Cervoni, 1978; Marcoullis et al., 1978; Welch and Dobson, 1978; Geerts et al., 1981c; Craig and Rickard, 1981). Craig and Rickard (1981) used the method to purify antigens from crude somatic and cyst fluid extracts prepared from T. saginata, T. hydatigena and Echinococcus granulosus. They used sera obtained from infected animals for the preparation of their immunoaffinity columns. It must be anticipated that eluates from such columns contained a mixture of antigens, some specific for the parasite and others which are shared with other parasites. This procedure, nevertheless, resulted in improved specificity in their tests. Geerts et al. (1981c) used specific antibodies to purify an antigen from T. saginata referred to as Fraction 10. This antigen fraction proved not to be specific for T. saginata, and was found to crossreact with metacestodes of E. granulosus and T. hydatigena. In the present study "Fraction 10" of Geerts et al. (1981c) was found to be partially identical to Antigen 4. Since this antigen has also been shown to be widely distributed in different

parasites, it is unlikely to provide adequate specificity in tests for bovine cysticercosis.

Preparative isoelectric focusing may be a useful initial step in the isolation of Antigen 4, 8, and 11 since these three antigens have been shown to have different pIs. However, the pIs of these antigens are fairly close, that is 5.21, 6.95 and 5.50 for Antigen 4, 8 and 11, respectively. Isoelectric focusing may therefore not be a method of choice for the purification of these antigens. Affinity chromatography is therefore necessary in the purification process. From these results, it is therefore not surprising to note that the attempts to purify T. saginata antigens using DEAE-cellulose ion exchange chromatography failed to provide specific antigens.

The specificity of the three antigens in the detection of bovine cysticercosis in naturally and experimentally infected animals is fully discussed in Chapter III.

CHAPTER III

IMMUNODIAGNOSIS OF BOVINE CYSTICERCOSIS

1. INTRODUCTION AND REVIEW OF LITERATURE

Light infections of bovine cysticercosis can easily escape detection at routine incision of the muscles of mastication, tongue, heart, oesophagus, diaphragm and triceps brachii, which are commonly considered predilection sites (Mann and Mann, 1947). Viljoen (1937) examined 113 lightly infected cattle and found that 11.5% of these animals did not harbour any cysts in these sites. Dewhirst et al. (1967) showed that routine meat inspection can pass one out of every four infected cattle as fit for human consumption. More recently, Walther and Koske (1980) examined 79 calves originating from a known T. saginata endemic area. The conventional meat inspection procedures could detect only 38.3% of the infected animals. Total dissection of the whole carcasses revealed a 75.9% infection rate, while 56.7% of lightly infected animals had no cysts in the predilection sites.

Several antemortem immunodiagnostic procedures have been developed for bovine cysticercosis, and several of these have been applied as an adjunct to meat inspection procedures. The literature on these immunodiagnostic tests has been reviewed extensively by Geerts et al. (1977) and Kamanga-sollo, (1981). The following are the tests which have been employed:

1.1 The complement fixation tests (CFT)

The CFT has been used by numerous workers in the diagnosis of bovine cysticercosis (Clarenburg, 1932; Soulsby, 1963; Frick and Susse, 1970; Susse and Frick, 1970; Lamina and Hein, 1970; Martin, 1972; Omarov, et al. 1973).

The CFT has been shown to be more sensitive than the indirect haemagglutination test, but less specific (Frick and Susse, 1970). The greatest shortcoming of this test is that it can only detect antibodies during a limited period of infection in calves and false negative reactions are frequently recorded in infections of animals older than six months. Therefore, this test is unreliable for the diagnosis of bovine cysticercosis (Lamina and Hein, 1970). Enyenihi (1974) suggested that this may be due to a change in the type of immunoglobulin produced by the host at different stages of infection. He further suggested that research should be done to elucidate the dynamics of the production of complement fixing antibodies in the bovine host.

1.2 The immunoprecipitation tests (IP)

Gel precipitation is one of the fastest and easiest methods to perform, but it is very unreliable (Frick and Susse, 1970; Aksenova, 1973; Gallie and Sewell, 1974a, b, 1976). The technique can detect only heavily infected animals (Frick and Susse, 1970; Grossklaus and Walther, 1970, 1971).

The lack of sensitivity of this test prohibits its use in the antemortem diagnosis of bovine cysticercosis (Mosina, 1965; Cramer and Dewhirst, 1965).

1.3 Indirect fluorescent antibody tests (IFA)

Calamel and Soule (1972) screened a variety of specific and non-specific antigens for their suitability in the IFA test. They found that cryostat sections of the activated oncospheres of T. saginata gave the most satisfactory results. Several other research workers have used oncospheres of T. saginata in IFA (Soule et al., 1971; Machnicka, 1973; Rydzewski et al 1975; Gathuma et al., 1978).

Non-specific fluorescence giving rise to false positive reactions has been observed by many workers using this test (Grossklaus and Walther, 1971; Wilkerhauser et al., 1974). In contrast, Euzeby and Dubra (1970) succeeded in diagnosing 100% of cases of bovine cysticercosis in 20 slaughter cattle using cryostat sections of T. saginata. The discouraging results found by Grossklaus and Walther (1971) in their IFA tests, however, led them to reject this test as a mean of diagnosing bovine cysticercosis. Soule et al. (1972) have suggested the use of a more sensitive soluble antigen fluorescent antibody test (SAFA) as an alternative to IFA. The recent development of enzyme immunoassays (EIA) appears to be a promising approach as it is easier to perform with simple equipment without reduction in sensitivity.

1.4 The immunoelectrophoresis (IEP) and counterimmunoelectrophoresis (CIEP) tests.

There are a few reports on the use of IEP and CIEP in the diagnosis of bovine cysticercosis (Leikina et al., 1971; Machnika, 1974a; Soulsby and Sanquini, 1974; Geerts et al., 1979; 1981a).

Geerts et al. (1979) analysed the antigenic components of T. saginata proglottides in IEP. They defined 22 antigenic components but most of these were of host origin and were not specific for the cestodes. The same workers also observed that calves experimentally infected with T. saginata eggs showed 3 precipitin lines, depending on the evolutionary stage of the infection. The precipitating antibodies could be detected 3 weeks post infection, but the major drawback was the high number of false negative reactions in animals with less than 50 cysts.

CIEP for the serodiagnosis of cysticercosis in cattle has been evaluated by Soulsby and Sanguini (1974). They used sera from experimentally and naturally infected animals and antigens from the adult and the larval stages of T.saginata and larval stages of T.crassiceps, prepared by the method of Kent (1963). Encouraging results were obtained with the CIEP technique with sera obtained from naturally infected cattle showing light (1-4 cysts), moderate 5-20 cysts), and heavy (over 20 cysts) infections as assessed by carcass inspection. The CIEP test permitted a classification of the degree of infection based on the number and intensity of precipitin lines

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observed. However, false positive reactions were observed with sera from adult normal cattle. These false positive reactions posed problems similar to those encountered with other sero-diagnostic tests.

1.5 The intracutaneous tests (ICT)

Homologous as well as heterologous antigens have been employed in the intracutaneous tests (Skrovtsov et al., 1941; Bugyaki, 1961; Froyd, 1963; Leikina et al., 1962, 1966; Graber and Thome, 1964; Dewhirst et al., 1960, 1967; Frick and Susse, 1970; Aksenova, 1973; Bratanov et al., 1974; Hilwig and Cramer,1982, 1983) There is generally poor agreement between various workers about the suitability of any particular antigen. For example, Bugyaki (1961) found that an extract of scolices gave the most reliable reactions, whereas Leikina et al., (1966) found that this antigen gave low specificity. Frick and Susse (1970) found that an extract of T. saginata gave more specific reactions. However, Bratnov et al. (1974) reported that an antigen prepared from T. saginata metacestode was more specific than those from the T. saginata whole worm extract.

Maximal intrademal reactions were observed within a few hours after injection of an allegen (Frick and Susse, 1970), but the reaction observed beyond 24 hours showed a better correspondence with the post-mortem findings. It can be concluded that although the sensitivity of the ICT tests is high, it could only be an attractive diagnostic method when highly purified and specific antigens are used.

1.6 The indirect haemagglutination test (IHA)

Antigens prepared from the proglottides of T. saginata or from the scolices and cyst membrane of T. saginata metacestodes have been used for the IHA test by various workers (Dewhirst et al., 1967; Alferova, 1969; Enyenihi, 1970; Frick and Susse, 1970; Omarov et al., 1973; Machnicka 1974b. Gallie and Sewell 1974a, b; Tailliez et al.,1976). Gallie and sewell (1974a, b) found that these antigens provided the same degree of sensitivity, while Tailliez et al. (1976) claimed that the aqueous extract of T. saginata was a more specific and sensitive antigen than the aqueous extract of T. saginata metacestodes.

False positive reactions are the major disadvantages of this test.

1.7 The latex agglutination test (LAT)

Extracts of T. saginata metacestodes are the most frequently used antigens in the LAT. Sokolovskaya and Moskvin (1967) compared the efficiency of an extract of T. saginata with an extract of the metacestodes, and confirmed that the T. saginata metacestode extract was a better antigen. On the other hand, Grossklaus and Walther (1970, 1971) obtained better results with a saline extract of T. saginata.

The LAT is considered unsuitable for the diagnosis of bovine cysticercosis due to the lack of specificity . (Enyenihi, 1970; Fillippov, 1971; Grossklaus and Walther, 1971; Alferova et al., 1972; Shekhovtsov et al., 1972).

1.6 The indirect haemagglutination test (IHA)

Antigens prepared from the proglottides of T. saginata or from the scolices and cyst membrane of T. saginata metacestodes have been used for the IHA test by various workers (Dewhirst et al., 1967; Alferova, 1969; Enyenihi, 1970; Frick and Susse, 1970; Omarov et al., 1973; Machnicka 1974b. Gallie and Sewell 1974a, b; Tailliez et al.,1976). Gallie and sewell (1974a, b) found that these antigens provided the same degree of sensitivity, while Tailliez et al. (1976) claimed that the aqueous extract of T. saginata was a more specific and sensitive antigen than the aqueous extract of T. saginata metacestodes.

False positive reactions are the major disadvantages of this test.

1.7 The latex agglutination test (LAT)

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1.8 Enzyme immunoassays (EIA)

Enzyme linked immunosorbent assays for the diagnosis of bovine cysticercosis have been performed by many workers (van Knapen et al.,1979a, b; Craig and Rickard ,1980; Harrison and Sewell, 1981a, b; Geerts et al. 1981a, b). The results obtained correlated remarkably well with those of IHA. But the major drawback of this test has been false positive results. This may be attributed to the crude antigens which have been used in this test (Craig and Rickard, 1980).

1.9. Concluding remarks on serodiagnostic tests

Lack of sensitivity and specificity has been a prominent feature of most of the immunodiagnostic tests mentioned above. Just as routine meat inspection has failed to detect low grade infection, serodiagnostic methods have failed to detect low levels of antibody produced in light infections (Craig and Rickard, 1981). Another problem in endemic areas is that of neonatal infection. These infections are very common and usually result in poor immune response to T.saginata antigens (Soulsby, 1963; Gallie and Sewell, 1974a; b).

It is apparent that crude parasite extracts have been used as antigens in most immunodiagnostic tests for bovine cysticercosis. This has resulted in difficulties with standardization of the procedures and interpretation of results (Chordi and Kagan, 1965; Oriol et al., 1971). Crude parasite extracts are complex mixtures of antigens some of which are confined to the parasite and others that are shared with other

infective agents (Kent, 1963). Purification of the appropriate antigens has been emphasised by many research workers, as the initial step towards the improvement of many diagnostic tests. (Geerts et al., 1977., Craig and Rickard, 1980) Nevertheless, recent studies on taeniid antigens suggest that a single purified antigen may be insufficient for serodiagnostic purposes, and that a complex mixture of antigens may be necessary to detect relevant antibody responses in bovine cysticercosis (Enyenihi, 1970, 1974; Geerts at al., 1979, 1981b).

Lack of specificity has also been attributed to the presence of cross reacting antigens. Cestodes are known to share many antigens with other parasites, such as trematodes and nematodes (Biguet et al., 1962; Capron et al., 1968; Enyenihi, 1970; Poletaeva, 1982).

The experiments described in this chapter were aimed at improving the specificity of two recognized sensitive immunoassays) namely radioimmunoassay (RIA) and enzyme immunoassay EIA) through the use of selected antigens of T. saginata metacestodes. These antigens (Antigens 4, 8, and 11), were selected on the basis of their uniform presence in T. saginata metacestodes and their rare occurrence in other common parasites of cattle (ChapterI). The studies described in Chapter II provided a characterization of these antigens in order to facilitate their isolation and purification.

The purpose of the investigations described in this chapter was to examine the serodiagnostic suitability of

Antigens 4, 8 and 11 in studies using sequential serum samples from experimentally infected calves, sera from naturally infected and non-infected animals and to compare the results with findings of conventional meat inspection procedures.

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MATERIALS

2. MATERIALS

2.1 Experimental animals

Three Friesian bull calves, 3 months of age, were purchased from the Faculty of Veterinary Medicine farm, Kabete. This facility is considered to be free of T.saginata cysticercosis. However, the presence of intestinal parasites was confirmed through faecal examination (Table 9).

The calves were eartagged with numbers 854, 867 and 870. Pre-infection bleedings were performed, and the calves were then dewormed at two week intervals using thiabendazole (Merck Sharp Dohme). The calves were kept in isolation for 5 weeks and dosed with approximately 300,000 T.saginata eggs with 82% viability as deterimed by the Silverman technique (Silvermann, 1954). The calves were bled at weekly intervals for 37 weeks. Calf No. 870 died of pneumonia 5 weeks post infection. A total dissection of the carcass was performed to establish the presence of T.saginata metacestodes

After 5 weeks, calves No.854 and 867 were returned to the University farm where they grazed with other calves for a period of 32 weeks.

2.2. Serum samples

2.2.1. Serum samples from experimental animals

Blood samples from the two remaining experimentally infected calves were obtained at weekly intervals for a period of 37 weeks. Serum was obtained as described in Chapter I.

Weekly serum samples were tested for the presence of antibodies to Antigen 4, 8 and 11 using enzyme immunoassay and radioimmunoassay (Chapter III, Section 2).

2.2.2. Hyperimmune sera

Hyperimmune sera from calves No.835, 846 and 900 were available from a previous study (Kamanga-Sollo, 1981). These calves had been immunized with homogenates of T. saginata and its metacestode as shown in Appendix II.

The serum samples were titrated in enzyme immunoassay,and preimmune serum samples from the same calves served as negative controls.

2.2.3 Specific antisera to Antigen 4, 8, and 11

Hyperimmune antisera specific for each Antigen 4 and 8, and one batch of goat antiserum to Antigen 11 were obtained by the immunization of rabbits and a goat with immunoprecipitates as described in Chapter I.

2.2.4 Goat-anti bovine IgG serum used in preparing an enzyme conjugate

A batch of goat anti-bovine IgG was obtained from ILRAD through the courtesy of Dr. A.J. Musoke. This was used for the preparation of a glucose oxidase conjugate as described in Chapter II.

2.2.5 Serum samples from other laboratories

A serum sample labelled RS 7 was obtained from an animal reared free of bovine cysticercosis, while a serum sample labelled RS 10 was obtained from an animal orally infected with T. saginata eggs. All these samples were obtained through the courtesy of Dr. P. Stevenson, Edinburgh Scotland.

Serum samples labelled M83 were collected at weekly intervals from a calf which had been reared at the Kenya Agricultural Research Institute, Muguga. The calf had been orally dosed with 50,000 eggs of T. saginata, and a total of 8,630 cysts had been recovered from the animal at slaughter. These samples were obtained through the courtesy of Mrs. J. Onyango-Abuje.

A pool of normal bovine serum from 3 animals labelled I84 was obtained from ILRAD through the courtesy of Dr. A.J. Musoke.

2.2.6 Serum samples from non-infected and naturally infected cattle

A total of 397 serum samples from naturally infected (205) and non-infected (192) were collected from cattle slaughtered at Kenya Meat Commission abbatoir, Athi River, and other slaughterhouses around Nairobi.

Each carcass was examined according to meat inspection regulations. The number of T. saginata cysts and their distribution in the body of the animal was recorded. The presence of other parasites was also recorded.

2.3 Antigens

Antigens 4, 8, and 11 obtained by affinity chromatography (Chapter II) were used as antigens in the sandwich enzyme immunoassay and the radioimmunoassay.

METHODS

3. METHODS

3.1 Sandwich enzyme immunoassay

The sandwich enzyme immunoassay was performed principally as described by Voller et at.(1976) and Voller, (1980).

The plate was either coated with rabbit antiserum specific for Antigen 4 or 8, or goat antiserum to Antigen 11, followed by the corresponding homologous antigen. The test samples from experimentally infected, naturally infected, or non-infected animals were then added. Goat anti-bovine IgG glucose oxidase conjugate was used as an indicator of binding of antibodies to the antigen captured on the plate.

3.1.2 Coating of plates for the assay of antibodies to T. saginata metacestodes

Falcon microtitre plates (Dickinson and Co, Oxnard, California, USA) were coated with either rabbit anti-Antigen 4 or 8 or goat anti-Antigen 11, using PBS coating buffer (Appendix IX) and following the method described by Lindqvist et al.(1982). The optimal dilution for coating was found to be 1/1000. The plates were placed in humid boxes for 18 hours at room temperature. Some of the plates were used the following day, and the rest were stored frozen at -20°C. When needed, the plates were thawed at 37°C, washed with PBS-Tween buffer (Appendix IX) and a homologous antigen added on the plate.

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3.1.3. Titration of antigens

A checker board titration was performed on plates coated with a corresponding antiserum to determine the optimal dilution of antigen to be used in the assay. Antigens purified by affinity chromatography were diluted in assay buffer (Appendix 1X) to make a final protein concentration of 25 ug/ml, 50 ug/ml and 100 ug/ml as determined by the method of Lowry (Lowry et al., 1951). One hundred microlitres of these dilutions were added to the plate, and after incubation for 1 hour at 37°C, the plate was washed with PBS-Tween buffer (Appendix 1X) and specific conjugate added as described in Chapter II. The optimal antigen dilution was found to be 50 ug/ml. This dilution was therefore used in all subsequent assays.

3.1.4. Titration of conjugate

A checker board titration was performed to determine the optimal dilution of goat anti-bovine IgG conjugate for use in the final assay.

The plate was coated with serial dilutions of bovine serum ranging from 10^{-2} to 10^{-6}. The conjugate was diluted from 1/200 to 1/25,600. A dilution of 1/800 of the conjugate was found to be optimal.

3.1.5. Titration of positive and negative sera

A checker board titration was performed using plates coated with specific antisera followed by 50ug of purified antigen per ml.

Table 9. Optimal conditions for the "sandwich" enzyme immunoassay

	Dilution	Volume	Reaction Time
Dilution of rabbit or goat-antiserum to specific antigen for coating of plates	1/1000	100ul	18 hours Room Temperature
Dilution of antigen	50 ug/ml (Protein)	100 ul	1 hour
Test dilution of serum samples	1/400	100ul	18 hours, Room Temperature
Dilution of goat anti-bovine IgG - glucose oxidase conjugate	1/800	100ul	1 hour (37^{o}C.)

Preinfection serum samples from calves No. 835, 846, 854, 867, 900, and samples RS 7 and I 84 were used as negative controls. Hyperimmune sera from calves No. 835 and 846, immunized with T. saginata metacestode extract and calf No. 900 immunized with T. saginata crude saline extract (Kamanga-Sollo, 1981) and experimentally infected calves No. 854 and 867 were used as positive controls.

The serum dilution giving maximum optical density difference between positive and negative serum samples was selected as a working dilution suitable for screening tests.

3.1.6. The assay

Sandwich enzyme immunoassay was performed on plates coated as described in 3.1.2. The optimal dilution of various test reagents are summarised in Table 9. The test was performed essentially following the steps described by Voller et al., (1976) and Voller (1980). The sera from experimentally infected animals (No. 854, 867 and M83) were titrated in order to obtain antiboby titres to Antigens 4, 8 and 11. The antibody titres were expressed as decribed by de Savigny and Voller, (1980).

3.2 Radioimmunoassay

The same material used in the enzyme immunoassay were also used in the radioimmunoassay, except that Antigen 4 was excluded from the RIA.

3.2.1 Labelling of antigens

The protein concentrations of Antigens 8 and 11 were adjusted to 300ug/ml and 360ug/ml, respectively. The chloramine T method of labelling antigen using ^{125}I was performed as described by Hunter (1979).

3.2.2. The assay

The radioimmunoassay was performed essentially following the procedure described by Musoke et al., (1981). Briefly, the following proportions of reagents were used: normal bovine serum diluted 1:10 (20 ul); ^{125}I-labelled antigen (20 ul) sera from experimentally infected animals or naturally infected animals diluted 1:100 (20 ul). The samples were diluted in TEN-buffer (Appendix X). The mixture was incubated at $4^{o}C$ for 3 hours before the addition of 50ul of goat anti-bovine IgG serum. The reactants were further incubated at $4^{o}C$ overnight. The resultant precipitates were washed 3 times with 500 ul of TEN-buffer and their radioactivity measured in a Packard 5360 Auto-Gamma Scintillation Spectrometer.

In the RIA, the serum sample was considered positive if the bound radioactivity was found to be above or equal to twice the background count (Nantulya et al., 1979). The preinfection serum samples and negative control samples described in section 3.1.5 were used to provide the mean background count of the assay system. Hyperimmune sera No.835, 846, 900, RS10 and sera from experimentally infected animals No.M83, 854 and 867 served as positive controls.

3.3. Evaluation of results

The following terminology will be used in the evaluation of results:

(i) Positive/Negative ratio (P/N), (ii) Sensitivity, (iii) Specificity, and (iv) Predictive value.

Students t-test based on the McNemar method of correlated proportions was used in the statistical evaluation of results (McNemar, 1955.; Thorner and Remein, 1961.; Martin, 1977).

3.3.1. Definition of Positive/Negative ratio (P/N)

P/N ratio is an expression of absorbance units obtained from a simultaneous test on the infected serum as compared to the reference negative serum. P/N ratio is expressed by the formula.

$$P/N = \frac{\text{OD, test serum sample}}{\text{OD, negative reference serum sample}}$$

In this study, eight negative serum samples whose mean ODs $\pm$ SD are given in Table 12, were regarded as negative controls at a test dilution of 1/400. The P/N ratio of all field samples was calculated on the basis of the average OD of known negative samples. P/N ratios greater than 2 was regarded as positive (de Savigny and Voller, 1980).

Table 10. Mean OD of 8 negative Control serum samples used in the calculation of P/N values

Identification of preimmune serum samples	OD at 410 nm OD of duplicate sample	Mean ± SD
No: 835	0.12	
No. 846	0.15	
No: 854	0.34	
No: 867	0.46	0.31 ± 0.14
No. 870	0.49	
No: 900	0.20	
No: RS7	0.33	
No: I 84	0.35	

3.3.2. Definition of sensitivity, specificity and predictive value

Sensitivity of the test is defined as ability of the test to correctly detect an animal with a specified disease (Vecchio, 1966; Martin 1977). This definition of sensitivity must be differentiated from its common usage, which is the ability to detect small amounts of antigen or antibody (Brinley-Morgan, et al., 1973). Specificity is the ability of the test to correctly detect healthy animals, i.e. animals not having the specified disease. Predictive value of the test is defined as the proportion of test positive animals which are diseased (Vecchio, 1966.; Katz, 1974.; Stewart, 1974). The predictive value is a function of sensitivity, the false positive rate and the prevalance of the disease (Vecchio, 1966.; Martin, 1977).

For the evaluation of sensitivity and specificity of the enzyme immunoassay, the data were arranged in a four-fold classification as shown in Table 12. Each antigen was evaluated separately for its ability to detect infection with T. saginata cysticerci.

3.3.3. Statistical evaluation of sensitivity and specificity of enzyme immunoassays (EIA) and radioimmunoassays (RIA)

Student's t-test was used to determine whether there were any statistically significant differences between sensitivities and specificities of the EIA and RIA using Antigens 8 and 11. The test was carried out following

McNemar's method for corrected proportions (McNemar, 1955) as described by Thorner and Remein (1961) and Martin (1977).

The results of EIA and RIA were arranged in a four-fold classification as shown in Table 12.

Table 11. A four-fold classification for calculating sensitivity and specificity using data obtained from postmortem diagnosis and immunoassay.

Immunoassay Results	Postmortem diagnosis Positive	negative	Total
Positive	a	b	a + b
Negative	c	d	c + d
Total	a + c	b + d	a + b + c + d =n

Legend:

a) Animals with bovine cysticercosis detected by the test (true positives)

b) Animals positive in the test, but negative at postmortem inspection (false positives)

c) Animals negative to the test but found infected with T. saginata cysticerci at postmortem (false negatives)

d) animals without bovine cysticercosis

$$\% \text{ sensitivity} = \frac{a}{a + c} \times 100$$

$$\% \text{ specificity} = \frac{d}{b + d} \times 100$$

$$\% \text{ predictive value} = \frac{a}{a + b} \times 100$$

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b) Animals positive in the test, but negative at postmortem inspection (false positives)

c) Animals negative to the test but found infected with T. saginata cysticerci at postmortem (false negatives)

d) animals without bovine cysticercosis

$$\% \text{ sensitivity} = \frac{a}{a + c} \times 100$$

$$\% \text{ specificity} = \frac{d}{b + d} \times 100$$

$$\% \text{ Predictive value} = \frac{a}{a + b} \times 100$$

Table 12. A four-fold classification of data obtained from radioimmunoassay and enzyme immunoassay for in determining differences in sensitivity and specificity

Enzyme immunoassay results	Radioimmunoassay Positive	Negative	Total
Positive	e	f	e+f
Negative	g	h	g+h
Total	e+g	f+h	e+f+g+h = n

If the two tests are equally sensitive, the proportions:

$$\frac{f}{f+g} \quad \text{and} \quad \frac{g}{f+g}$$

should each equal to 0.5. If either of these proportions varies from 0.5 by an amount greater than can be attributed to sampling variation, the two sensitivity proportions can be considered significantly different. The hypothesis (Ho) then is: $\frac{f}{f+g} = \frac{g}{f+g}$

The alternative hypothesis (H_2) is: $\frac{f}{f+g} \neq \frac{g}{f+g}$

This hypothesis was tested by t test using the formula:

$$t = \frac{\left(\frac{f}{n} - \frac{g}{n}\right)}{\sqrt{\frac{f+g}{n^2}}}$$

Table 12. continued on next page.

Table 12. (Cont'd)

A t value of 1.96 or more was indicative of a significant difference in sensitivity proportion at the 5 percent probability level.

When f+g was equal to or less than 20, a correction for continuity was made by subtracting $\frac{1}{n}$ from the numerical value of the the numerator.

The t value was then calculated using the formula:

$$t = \frac{\left(\frac{f}{n} - \frac{g}{n}\right)\frac{1}{n}}{\sqrt{\frac{f+g}{n^2}}}$$

For comparison of specificity, the results of enzyme immunoassay and radioimmunoassay were arranged in a similar four fold classification table, but only those samples found to be negative for bovine cysticercosis at postmortem diagnosis were compared.

RESULTS

4. RESULTS

4.1. Recovery of T. saginata cysts from experimentally infected calves.

Calf No.854 and 867 remained healthy and were slaughtered 37 weeks after infection, while calf No. 870 died of pneumonia 5 weeks after dosing with eggs. A total carcass dissection was performed on all calves. Calf No. 870 was found to harbour 21 cysts and calf No. 854 had 25 viable cysts with one degenerating cyst in the heart, and calf No. 867 had 28 viable cysts.

There was no particular predilection site for the cysts. With exception of calf No. 854, none of the cysts was found in the so called predilection sites, namely the tongue, oesophagus, diaghragm, muscles of the head of triceps brachii.

4.2. Enzyme immunoassay results

4.2.1 Antibody titres to Antigen 4, 8 and 11 in experimentally infected cattle

Figure 21 shows how the enzyme immunoassay antibody titres were determined. This method was adopted from the work described by de Savingy and Voller (1980). In general, there was a variable antibody response to the various antigens, with the response to

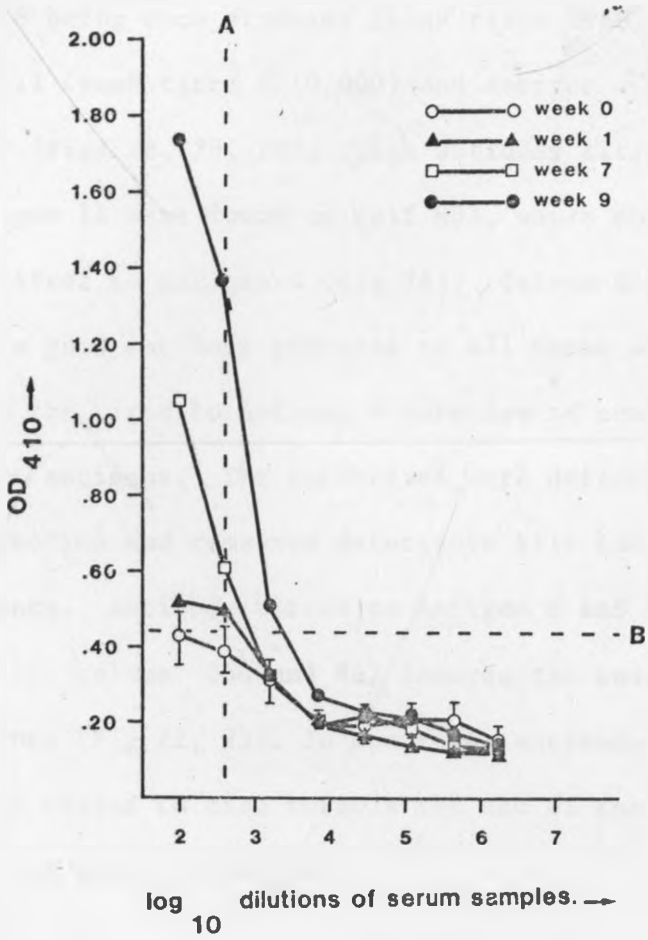


Fig.21: Illustration showing the method for determination of antibody titres by sandwich enzyme immunosassays using data obtained with Antigen 8 as an example; using serum samples from calf no. 854.

Titre = dilution of serum giving cut off value.

A = Indicates absorbance value at test serum dilution 1/400.

B = Intercept with horizontal line indicating the titre.

Method described by de Savingy and Voller (1980).

Antigen 8 being much stronger (peak titre 1:80,000) than to Antigen 11 (peak titre 1:10,000) and Antigen 4 (peak titre 1:1,500) (Figs 22, 23, 24). High antibody titres to Antigen 8 and Antigen 11 were found in calf M83, while this calf gave the lowest titres to Antigen 4 (Fig 24). Calves 854 and 867 mounted a good antibody response to all three antigens, although the tires to Antigen 4 were low as compared to the other two antigens. The antibodies were detected one week post-infection and remained detectable till the end of the experiments. Antibody titres to Antigen 8 and 11, showed a decline in calves 854 and 867 towards the end of the experiments (Fig 22, 23). In contrast, antibody titres to Antigen 4 tended to rise towards the end of the experiments in calves 854 and 867 (Fig 24).

4.3. Enzyme immunoassay results obtained with serum samples from non-infected and naturally infected cattle.

Table 13 shows an example of how sensitivity and specificity of the enzyme immunoassays were calculated. In the enzyme immunoassay using Antigen 8, a sensitivity of 66%, a specificity of 40% and a predictive value of 56% were found. When Antigen 11 was used, a sensitivity of 55% and a specificity of 48% were recorded, while the predictive value was 52%. When Antigen 4 was used, a sensitivity of 48%, a specificity of 40% and a predictive value of 46% were recorded.

Table 13. Evaluation of Enzyme immunoassay results using Antigen 8

Enzyme immunoassay Results	Postmortem diagnosis Positive	Negative	Total
Positive	132	104	236
Negative	67	69	136
Total	199	173	372

Sensitivity: $\frac{132}{199} \times 100 = 66\%$

Specificity: $\frac{69}{173} \times 100 = 40\%$

Predictive value: $\frac{132}{236} \times 100 = 56\%$

There was no statistically significant difference between the sensitivities obtained with Antigen 8 and 11 ($t=1.925$; $P > 0.05$). However, Antigen 8 gave a higher sensitivity than Antigen 4 ($t=2.528$; $P < 0.05$). A comparison between Antigen 11 and 4 also showed a significant differene in sensitivities ($t=5.885$; $P < 0.05$) with Antigen 11 giving a higher sensitivity value than Antigen 4.

There was no statistically significant difference between the specificities obtained when Antigen 8 was compared with Antigen 11, ($t=0.980$; $P > 0.05$), while there were statistically significant differences in specificities shown by Antigen 8 as compared to Antigen 4. Antigen 8 gave a higher specificity than Antigen 4 ($t=3.291$; $P < 0.05$). Antigen 11 was more specific than Antigen 4 ($t=2.297$; $P < 0.05$).

The specificity of enzyme immunoassay was low due to a high proportion of false positive results. However, most of the serum samples which gave these results were from animals which had calcified lesions in the liver.

4.4 Radioimmunoassay results obtained with serum samples from experimentally infected, non-infected and naturally infected animals

Figure 25 shows the results of radioimmunoassay using Antigen 11 and sera from experimentally infected animals. These results compare well with those obtained with the enzyme immunoassay in terms of variations in antibody levels in all three experimental animals.

Calf M83 showed a higher antibody response compared to animals 854 and 867. Antigen 8 gave results similar to those obtained with Antigen 11 (Fig 26).

Radioimmunoassay using Antigen 8 in assays performed with sera from naturally infected animals gave a sensitivity of 82%, a specificity of 25% and a predictive value of 58%. Antigen 11 gave a sensitivity of 76%, a specificity of 30% and a predictive value of 58%. A comparison of these results is given in Table 15. There was no significant difference in specificities of the radioimmunoassay using antigens 8 and 11 for the detection of antibodies ($t=0.741$; $P > 0.05$).

However, the sensitivity of radioimmunoassay was much higher than that of enzyme immunoassay ($t=6.812$; $P < 0.05$), while the specificity of radioimmunoassay was significantly lower than enzyme immunoassay ($t=6.976$; $P < 0.05$).

Table 14. Compararison of sensitivities, specificities and predictive values obtained using Antigen 4, 8 and 11 in enzyme immunoassay of serum samples from non-infected and naturally infected cattle as determined by meat inspection

	% Sensitivity	% Specificity	% Predictive value
Antigen 4	48	40	46
Antigen 8	66	40	56
Antigen 11	55	48	52

Table 15. Comparison of sensitivites, specificities and predictive values obtained using Antigen 8 and 11 in radioimmunoassay of serum samples from non-infected and naturally infected cattle as determined by meat inspection

	% Sensitivity	% specificity	% Predictive value
Antigen 8	82	25	58
Antigen 11	76	30	58

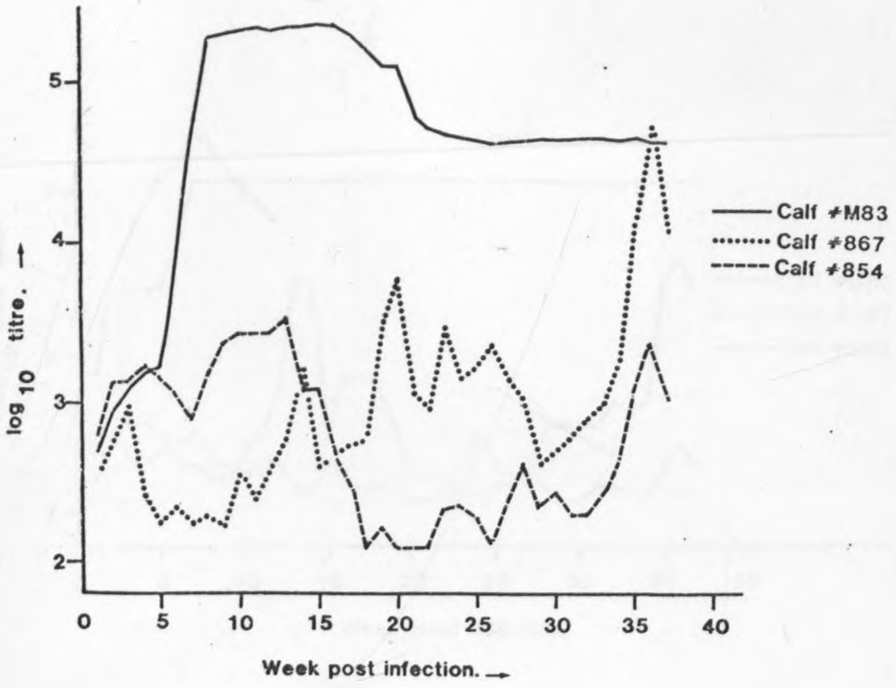


Fig. 22. Antibody responses to Antigen 8 induced in calves No. M83, 867 and 854, experimentally infected with eggs of T. saginata

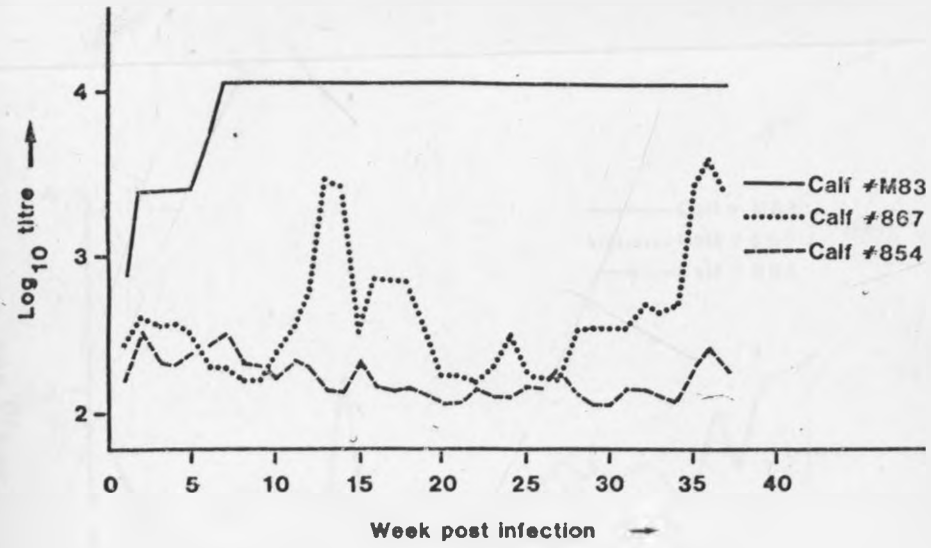


Fig. 23: Antibody responses to Antigen 11 induced in calves No. M83, 867 and 854 experimentally infected with eggs of T. saginata.

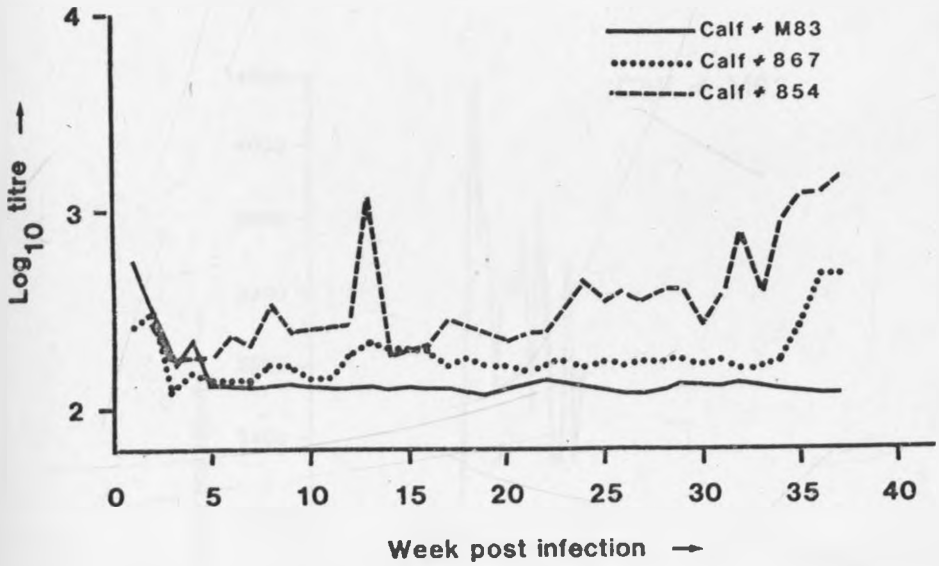
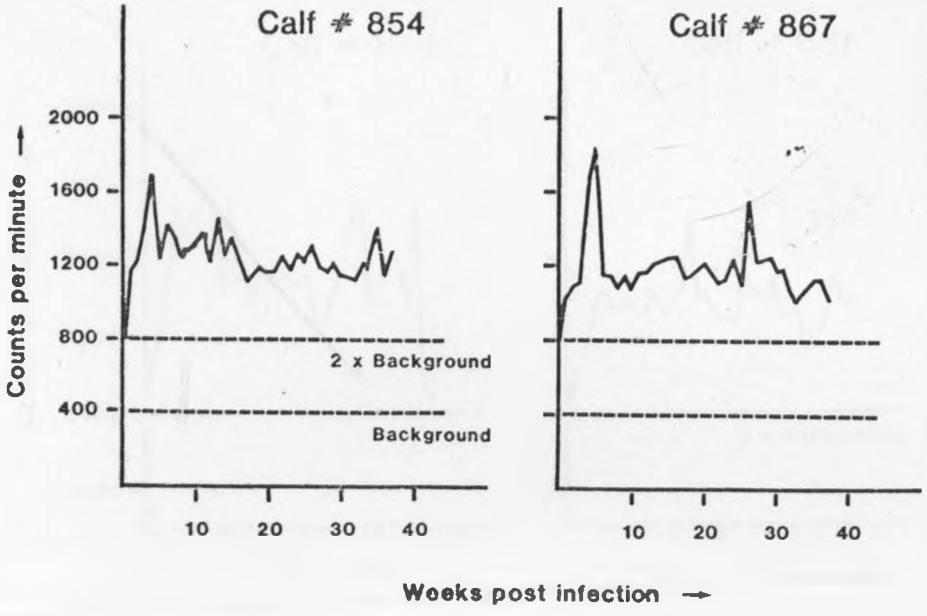


Fig. 24: Antibody responses to Antigen 4 induced in calves No. M83, 867 and 854 experimentally infected with eggs of T. saginata



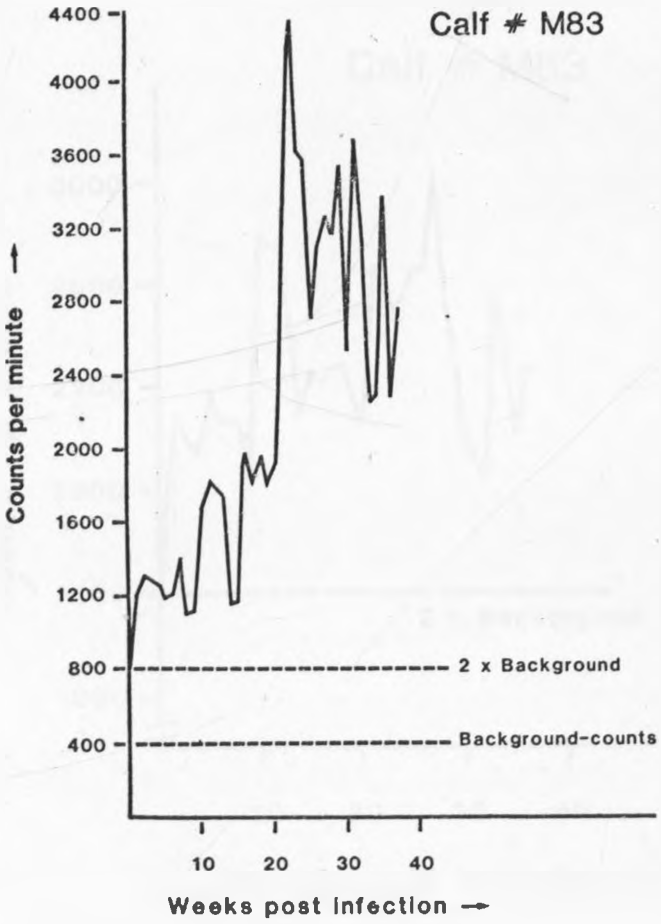
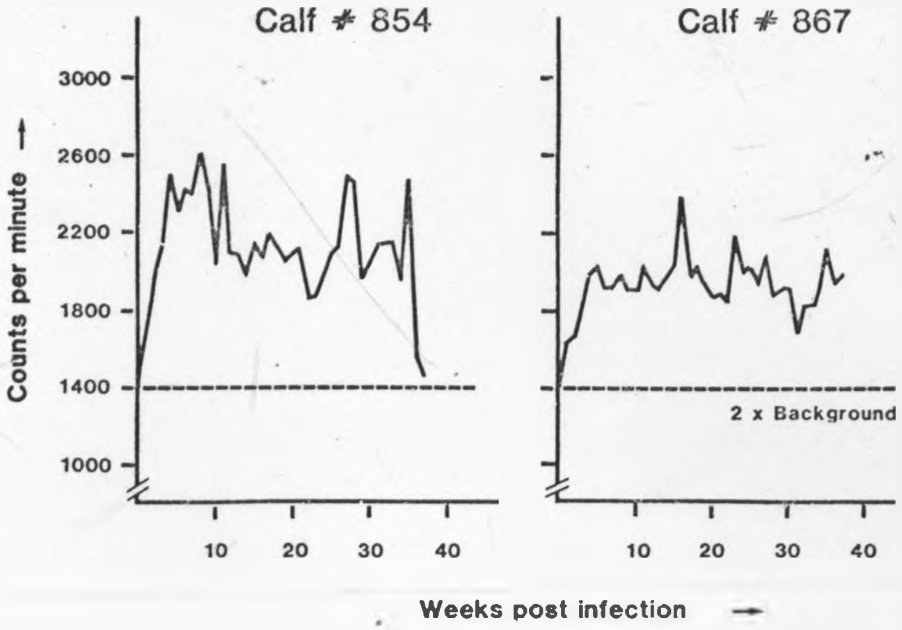


Fig. 25: Antibody response to Antigen 11 in calves No. 854, 867 and M83.



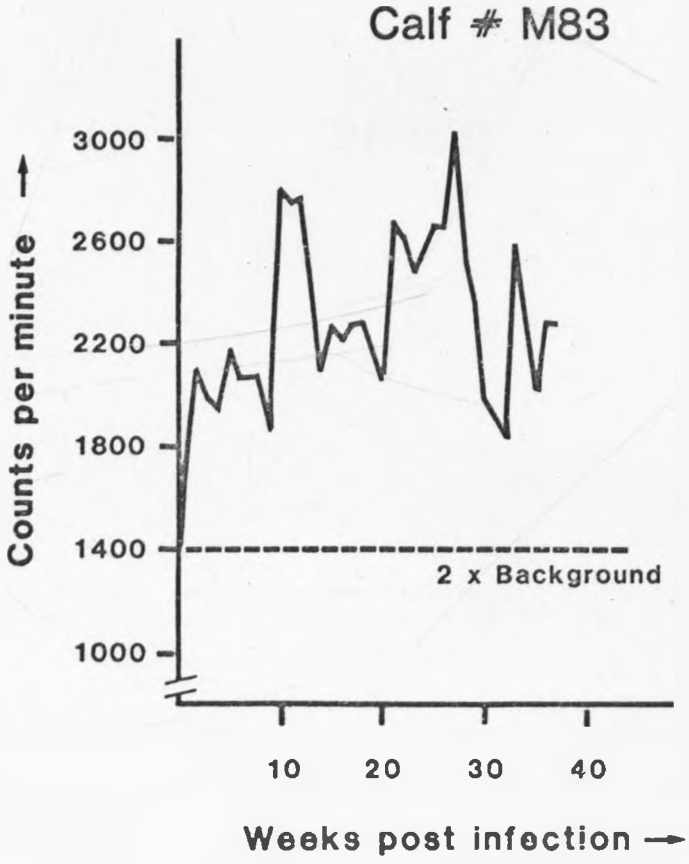


Fig. 26. Antibody response to Antigen 8 in calves No. 854,867 and M83.

DISCUSSION

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DISCUSSION

The establishment rate of cysts after exerimental infection was similar to that of Gathuma (1977), who obtained a rate of between 0.044% and 0.187% when he dosed calves with 50,000 to 150,000 eggs. These results mimic field conditions, where massive infection with T. saginata cysticerci is rare. In contrast, Gallie and Sewell (1974a, b, 1983) obtained 1.5 to 13.7% establishment rate when they dosed calves with 5,000 to 150,000 T. saginata eggs.

The difference in the establishment rate of cysts could perhaps be explained on the basis of the origin of calves. Whereas the calves used by Gallie and Sewell (1974a, b, 1983) came from a non-endemic area, Gathuma's (1977) calves, and those used in the present study were from areas with a high prevalance of bovine cysticercosis and they were also infested with other common parasites such as Moniezia spp. The possibility therefore exists that the calves were infected with parasites which share protective antigens with T. saginata. On other hand, the calves could have been from cows which had been infected with T. saginata metacestodes, but it is unlikely that they would have retained efficient passive immunity up to the age of 3 months. However, Onyango-Abuje (personal communication, see also Chapter III, Section 2.2.5) obtained an infection rate of 17.3% in experimentally infected calf M83, inspite of her animal coming from a hyperendemic area.

Antibody responses to Antigens 4, 8, and 11 were detected as

early as one week post infection in all experimentally infected calves. This finding is different from that of Gallie and Sewell (1974a, b) who could not detect anti-T. saginata antibodies in experimentally infected calves until 5 weeks post infection. Gathuma (1977) also observed that only 2 out of 8 experimentally infected calves had antibodies to T. saginata after one week of infection. A possible explanation for this difference is that in the studies reported in this thesis purified antigens as well as methods possessing high sensitivities were used. These results are very similar to those obtained with Antigen 11 in a previous study, where solid phase radioimmunoassay was used as the assay system (Kamanga-Sollo, 1981).

The sensitivities of the enzyme immunoassay and the radioimmunoassay were fairly good in the diagnosis of bovine cysticercosis cases as compared to the sensitivities of enzyme immunoassays obtained by several other investigators (Craig and Rickard, 1980; Geerts et al., 1981b). For example, Geerts et al., (1981b) recorded a sensitivity of 37.5% in naturally infected cows as contrasted with sensitivities of 66% and 55% for Antigens 8 and 11 obtained in this study. The improvement in sensitivity in this investigation may be attributed to the use of antigens with defined specificity, as opposed to Geerts et al., (1981b) who used crude extracts of T. crassiceps metacestode. However, the specificity of the enzyme immunoassay is low. The increase in sensitivity of the radioimmunoassay was accompanied by a decrease in specificity. There are

several reasons for this discrepancy. One of the reasons may be attributed to the cut-off point of 2 times the background counts used in the radioimmunoassay. This level was decided upon on the basis of the results of experimentally infected calves and also following similar assays carried out by other research workers (Nantulya et al., 1979). On this basis, the cut-off point appears justifiable. The reasons for increased sensitivity may have been due to the fact that in the radioimmunoassay the antigen was labelled as contrasted to the labelling of goat anti-bovine IgG in the enzyme immunoassay. This might suggest that the two test systems, using the same antigen, depend on different epitopes, one of which is more specific than the other. On the other hand, we could have increased the cut-off point, which would have resulted in improvement of the specificity but with a concomitant decrease in sensitivity (Thorner and Remein 1961; Williams 1982).

False positive results have generally been blamed on the presence of cross-reacting antigens in the crude preparations used in immunodiagnostic tests. However, in this study, purified, specific antigens were used, and the presence of cross-reacting antigens can be ruled out. It is tempting to suggest that the false positive results have been due to the inherent inadequacies of the standard meat inspection procedures which generally fail to detect light infections. Alternatively, the animals may have been infected with senescent eggs of T. saginata, which may have failed to develop into full cysts, although capable of inducing a detectable and persistent serological response.

It is conceivable that a single antigen, although specific, may not suffice for the consistent detection of antibodies to T. saginata metacestodes. A mixture of several antigens of adequate specificity and used in a sensitive immunoassay, is likely to be required for the detection of antibody responses to this and other parasites.

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CHAPTER IV

GENERAL CONCLUSIONS

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One of the classical concepts in parasite immunology is that parasites possess a very complex mosaic of antigens (Capron and Dessaint, 1982). The host immune response to the parasite is in turn a reflection of the diversity of the expression of parasite antigens. This diversity may depend on antigen presentation at the parasite level. For example, soluble antigenic components, such as excretory-secretory products may differ from antigens released during lysis or death of the parasite. These antigens may be different from the membrane-bound antigenic determinants.

The objective of the work reported in this thesis was to identify, isolate and characterise antigens that could be used in the serodiagnosis of bovine cysticercosis. Fifteen antigens of T. saginata metacestodes were defined using a reference pattern based on Laurell's crossed immunoelectrophoresis. The application of this method for the elucidation of the antigenic complexity of T. saginata metacestodes permitted investigations of the relationship between antigens from various parasites, and greatly facilitated the identification of antigens possessing potentially adequate specificities for use in serodiagnostic tests for bovine cysticercosis

It was shown in this study that several antigens of the T. saginata metacestode are shared with some commonly occurring parasites of domestic animals. Nevertheless, a few antigens were largely confined to T. saginata metacestodes.

Examples of such antigens are those which in this study were identified as Antigens 4, 8 and 11.

Antigens 4, 8 and 11 were found to have molecular weights of 63 kd, 260 kd and 68 kd respectively, based on SDS-PAGE analysis. Antigen 8 appeared to be a multichained antigen which consists of components with molecular weights of 110 kd, 72 kd and 61 kd. All these components gave reactions of identity in immunodiffusion tests, showing that they share common antigenic determinants.

Antigen 4 was found to be common in cestodes, and was found to be identical to an antigen referred to as Fraction 10 of T. saginata isolated by (Geerts et al., 1981c). Antigen 8 appeared to be unique to T. saginata and its metacestodes, since it was not found in any of the 30 different parasites examined in this study. Antigen 11 was found to be present also in the metacestodes of T. solium and in T. hydatigena and its metacestodes. Antigen 4 and 11 may therefore be the antigens responsible for the cross reactions between T. saginata metacestodes and other cestodes described by several authors (Williams 1979; Craig and Rickard, 1981).

Antigens 4, 8 and 11 have pI values of 5.21, 6.95 and 5.20 respectively. Purification of these antigens was achieved through a combination of molecular sieving, affinity chromatography and isoelectric focusing.

Little information is available concerning the kinetics of antibody production in cattle with bovine cysticercosis (Flisser et al. 1979). Some research workers have reported

that antibodies reach detectable levels approximately one month after experimental infection, and the titres are very variable depending on the assay system and the age of the calf (Wilkerhauser et al., 1974; Gallie and Sewell, 1974a, b). Considerable fluctuations in titres were also found in this study. All the three antigens elicited an early antibody response which could be detected one week after experimental infection. There was a strong antibody response to Antigen 8, in contrast to the low antibody titres to Antigen 4.

Evaluation of these antigens for use in the serodiagnosis of bovine cysticercosis, Antigen 8 and 11 had good sensitivity and specificity as compared to Antigen 4. The sensitivity and specificity of Antigen 4 was similar to that obtained by Geerts and his coworkers (1981a) when they used Fraction 10 of T. saginata.

When serum samples from animals assumed to be free from infection were examined in these test systems, there was a high rate of apparent false positive reactions. These may be attributed to the poor sensitivity of the meat inspection procedures which were presumed to provide the definitive diagnosis. But the apparent false positive reactions may have been due to abortive infections as the result of the ingestion of senescent eggs of T. saginata. Such eggs may fail to develop into mature cysts but nevertheless trigger an immune response and give rise to detectable antibody levels (Gemmell and Johnstone, 1977).

There are several ways in which the serodiagnostic procedures described in this study could be employed usefully. For example, the enzyme immunoassay using Antigen 8 and 11, could be used as an adjunct to the current meat inspection procedures. The immunoassays may be used reliably for the selection of animals which are heavily infected. These animals can then be separated for treatment or slaughtered at an early age, thereby saving the farmer from keeping animals which can be downgraded or condemned. The same method could be used to select animals which are to be taken to a feedlot for fattening.

Furthermore, those animals whose serum samples give positive serological reactions in spite of negative findings at routine meat inpection, could be regarded as lightly infected and their carcasses justifiably subjected to cold storage.

Results obtained so far indicate that cattle reared in an environment free from T. saginata, or cattle which upon total dissection of their carcasses have been found not to be infected, have consistently given negative reactions in serological tests using Antigens 8 and 11. Therefore, it could be advocated that serologically negative animals could be subjected to less vigorous meat inspection procedures aimed at detecting metacestodes of T. saginata

The detection of antibodies to T. saginata metacestodes in a group of animals would be an indirect way of identifying the human source of infection. Appropriate treatment of human carriers associated with cattle could be a valuable method in the control of this cyclozoonosis, at least in a limited area.

The present study has shown that even light infections can be detected in experimentally infected animals using Antigens 4, 8, and 11. Only of the 3 experimental animals was found at meat inspection to have a single cyst detectable by this method. Total carcass dissection of these 3 animals showed that each of these animals had 21, 25 and 28 cysts scattered in their musculature. All 3 animals would have been passed by the meat inspection as fit for human consumption.

In view of this, it is clear that the results obtained by testing serum samples from "non-infected" cattle using meat inspection procedures as the definitive parameter, are entirely unreliable. In the case of the 205 animals in which cysts were detected at meat inspection, 40 animals had less than 5 cysts, and 165 had more than 5 cysts at the obligatory incision sites. Thirty five of the 40 animals gave a positive reaction in both EIA and RIA, while all 165 animals with more than 5 cysts gave a positive reaction with Antigens 8 and 11.

Several explanations could be advocated for the negative serological results obtained in 5 of the 40 lightly infected animals. It is possible that an adjustment of the cut-off figure in the P/N ratio may have classified one or more of the 5 animals as positive. On the other hand, it is not surprising that some - or even many - animals fail to mount a detectable immune response to a few non-replicating cysts.

Cysticercosis of cattle cannot be compared with other infections, for example bacterial or viral, in which the organisms multiply and produce comparatively large amounts of antigens which usually are able to induce a strong immune response in the host.

The approach taken and the results obtained in this study have provided considerable insight in the immunological aspects of bovine cysticercosis. Further research is required to elucidate the possible practical application and consequences of these findings.

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APPENDIX I.

1. List of parasite extracts used in this study

Protein content of these extracts was determined by the method of Lowry et al., (1951)

Parasite extract	Source	Protein content mg/ml
T.saginata metacestode (IMS)	cattle	39.2
T.saginata	man	32.9
Moniezia spp	cattle	29.2
T.hydatigena	dog	43.9
T.hydatigena metacestode	goat & sheep	36.9
T. crocutae	hyena	33.5
T. crocutae metacestode	wildebeest	27.1
Hydatid cyst fluid	goat,sheep,cattle	45.8
T.multiceps metacestode	goat & sheep	8.7
T. solium metacestode	pig	25.8
Avitellina spp	cattle	32.9
Spirometra spp	hyena	110.0
Stilesia hepatica	goat,sheep,cattle	5.6
T. taenaiaeformis strobilocercus	mice	1.4
Dipylidium caninum	dog	23.8
Fasciola gigantica	goat,sheep,cattle	29.9

APPENDIX I continued on next page (164

Appendix 1 (cont'd)

Schistosoma bovis	cattle	8.6
Schistosoma mansoni	man	10.7
Paramphistomum spp	cattle	50.4
Paramphistomum spp	wildebeest	38.6
Oesophagostomum spp	Cattle.	37.3
Cooperia spp	goat,sheep,cattle	37.2
Setaria digitatum	cattle	19.9
Setaria equinum	zebra	9.1
Trichostrongylus spp	goat, cattle	15.2
Bunostomum spp	cattle,goat	31.0
Ancylostomum caninum	dog	10.0
Toxocara canis	dog	35.7
Ascaridia galli	chicken	28.3
Ascaris suum	pig	71.0
Haemonchus spp	cattle	26.5
Spirocerca lupi	dog	1.5

Appendix 1 (cont'd)

Schistosoma bovis	cattle	8.6
Schistosoma mansoni	man	10.7
Paramphistomum spp	cattle	50.4
Paramphistomum spp	wildebeest	38.6
Oesophagostomum spp	Cattle.	37.3
Cooperia spp	goat,sheep,cattle	37.2
Setaria digitatum	cattle	19.9
Setaria equinum	zebra	9.1
Trichostrongylus spp	goat, cattle	15.2
Bunostomum spp	cattle,goat	31.0
Ancylostomum caninum	dog	10.0
Toxocara canis	dog	35.7
Ascaridia galli	chicken	28.3
Ascaris suum	pig	71.0
Haemonchus spp	cattle	26.5
Spirocerca lupi	dog	1.5

APPENDIX II

2. List of antisera used in this study

Identification Animal species	Preparation used for immunization	Antisera prepared
Rabbit 104 Rabbit 115 Rabbit 131 Rabbit 140 Rabbit 158 Rabbit 176 Rabbit 79	T.sag.metacestode (IMS) Scolices of T.sag. metacestode	REFERENCE ANTISERUM: Rabbit anti-T. saginata metacestode serum
Rabbit 128 Rabbit 146 Rabbit 181	Inner fluid (F) of T.sag.metacestode	Anti-Inner Fluid serum
Rabbit 114 Rabbit 151 Rabbit 154	T. sag. homogenate	Anti-T.sag. serum
Rabbit 108 Rabbit 194 Rabbit 196 Rabbit 199	Precipitin line A (ref: Appendix III)	Anti-Antigen 8 serum
Goat 879	Precipitin line C (ref: Appendix III)	Anti-Antigen 11 serum
Rabbit 119 Rabbit 120	Precipitin line D (ref: Appendix III)	Anti-Antigen 10 serum
Rabbit 107 Rabbit 170 Rabbit 198	Precipitin line E (ref: Appendix III)	Anti-Antigen 4 serum

APPENDIX II continued on next page (166).....

Identification Animal species	Preparation used for immunization	Antisera Prepared
Calf 835	Inner fluid (F) of T.sag.metacestode	Calf Anti-Inner Fluid serum
Calf 846	T.sag.metacestode (IMS)	Calf Anti-Inner Membrane Scolex (IMS) serum
Calf 900	T.sag.homogenate	Calf Anti-T.sag. serum
Rabbit 152	T. crocutae metacestode	Rabbit Anti-T.croc. metacestode serum
Rabbit 174	T. crocutae homogenate	Rabbit Anti-T.croc. serum
Rabbit 139 Rabbit 166	Moniezia spp homogenate	Rabbit Anti-Moniezia serum
Rabbit 184	Fasciola gigantica homogenate	Rabbit Anti-F.gigant. serum

APPENDIX II continued
on next page (167).....

....APPENDIX II (cont'd)

Identification Animal species	Preparation used for immunization	Antisera prepared
Rabbit 138 Rabbit 149	Hydatid cyst fluid (HCF)	Rabbit Anti-HCF serum
[2]Rabbit 155 Rabbit 169	Antigen 13 of *T. saginata* metacestode	Rabbit Anti-Antigen 13 serum
[3]Rabbit	Precipitin line labelled Fr. 10 of *T. sag.*	Rabbit Anti-*T.sag.*Fr.10 serum

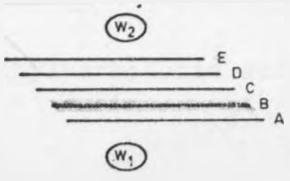
[1]Rabbit 79, anti-*T. saginata* scolices serum was obtained from Professor K.J. Lindqvist, of this department.

[2]Rabbit 155, anti-Antigen 13 serum was obtained from Dr. D.K. Gasangwa, formerly of this department.

[3]Rabbit anti-*T. saginata* Fr. 10 serum was obtained from Dr. S. Geerts, Institute of Tropical Medicine, Antwerp, Belgium.

APPENDIX III

Immunization scheme used to produce specific antisera to various components of T.saginata metacestodes.



KEY :

Antiserum well (W_1) : Pooled rabbit anti-T. saginata metacestode
Antigen well (W_2) : T. saginata metacestode (IMS).
A, B, C, D and E. : Precipitin line obtained in immunodiffusion (Kamanga-Sollo, 1981).

IMMUNIZATION OF RABBITS.

Precipitin line A, C, D and E were used to produce specific antisera to T saginata metacestode which were identified in Laurell crossed immunoelectrophoresis (CIE) as Antigen 8, 11, 10 and 4 respectively.

Precipitin line obtained in immunodiffusion	A	B	C	D	E	Antigen as defined in CIE
Rabbit Nº 108 194 196 and 199.	+	-	-	-	-	8
Goat Nº 879	-	-	+	-	-	11
Rabbit Nº 119, 120	-	-	-	+	-	10
Rabbit Nº 107 170 198	-	-	-	-	+	4

+ = rabbit or goat immunized with Antigen.
- = rabbit or goat not immunized with Antigen.

APPENDIX IV

4. List of buffers and solutions used in immunoelectrophoresis and immunodiffusion tests

4.1. Barbital/calcium lactate buffer for immunoelectrophoresis

4.1.1. For electrophoresis chambers:

Sodium barbital (Sodium 5,5-diethylbarbiturate)	105.1g
Barbital (5,5-diethylbarbituric acid)	16.6g
Calcium lactate	15.2g

Dissolved in 10 litres of distilled water. The pH was adjusted to 8.4 or 8.6.

4.1.2. For preparation of agarose gel

The above buffer was diluted with distilled water in the ratio of 2 parts buffer and 1 part water (v/v).

4.1.3. Phosphate buffered saline for preparation of agar gel for immunodiffusion (see Appendix IX)

4.2. Agar and agarose used in immunodiffusion and immunoelectrophoresis tests.

4.2.1. 1% agar in phosphate buffered saline, pH 7.4, for immunodiffusion tests:

Purified Oxoid Agar	2g
Phosphate buffered saline	200ml
Sodium azide	0.2g

4.2.2. 1% agarose in barbital/calcium lactate buffer with Triton X-100 and 1% polyethyleneglycol 6000 for crossed immunoelectrophoresis and immunoelectrophoresis.

Litex agarose (Type HSA)	3g
Barbital calcium lactate buffer	100ml
Distilled water	200ml
Triton X-100	150ul
Polyethyleneglycol 6000	3g

Before electrophoresis, Triton x - 100 was added to the antigen preparation to find concentration of 0.5%

APPENDIX V

5.0 Protein staining and destaining solutions

5.1. Coomassie Brilliant Blue 250 R solution:

Coomassie Brilliant Blue 250R	10g
Ethanol	900ml
Glacial acetic acid	200ml
Distilled water	900ml

5.2. Destaining solution for Coomassie Blue (5.1):

Ethanol	900ml
Glacial acetic acid	200ml
Distilled water	900ml

5.3. Ponceau S Solution:

Ponceau S	2g
1M acetic acid	1000ml
0.1M sodium acetate	1000ml

5.4. Destaning solution for ponceau S staining procedure:

3% (v/v) glacial acetic acid in distilled water

5.5. Crocein scarlet/Brilliant Blue stain:

Crocein scarlet	250mg
Brilliant Blue	15mg
5% acetic acid with 3% trichloroacetic acid	100ml

The dyes were dissolved by heating to 60^{o}C.

6.0 APPENDIX VI

6.1. Gel buffer (0.2M phosphate pH 7.0 with 0.2% SDS):

Sodium dihydrogen phosphate 1 monohydrate 17.64g

Di-sodium hydrogen phosphate 40.80g

Sodium dodecyl sulphate (SDS) 4.0g

Dissolve the salts in 1 litre distilled water and adjust the pH to 7.0 (with 0.1N acid or alkali) then make up to 2 Litres with distilled water. For use in electrophoresis dilute the buffer 1:2 (Buffer: Distilled water).

6.2. 5% Acrylamide stock solution:

Acrylamide 33.3g

Bis-acrylamide 0.9g

Dissolve the acrylamide reagents in 300mls of distilled water and filter through Whatman No.1 filter paper to remove insoluble material.

6.3. 70% Glycerol (w/v):

Glycerol (Sp. gr. 1.26)(BDH-Poole England)	55.6ml
Distilled water	44.4ml

6.4. 0.05% Bromophenol blue:

0.01g Bromophenol blue was dissoved in 20 mls distilled water.

6.5. 44mM phelylmethylsulphonyl fluoride (PMSF):

0.038g (PMSF) was dissolved into 5ml acetone/ethanol.

6.6. Coomassie Brilliant Blue 250R stain:

Coomassie Brilliant Blue 250R	1.0g
Methanol	452ml
Distilled water	452ml
Glacial acetic acid	96ml

The stain was filtered through Whatman No. 1 filter paper to remove insoluble material.

6.7. Gel destaining solution:

Glacial acetic acid	70ml
Methanol	200ml
Distilled water	730ml

6.8. 0.02% ammonium persulphate, analytical grade (Fisher Scientific Company, New York, USA):

0.12g ammonium persulphate was dissolved in 8 mls of distilled water.

6.9. N,N,N,'N-tetramethylenediamine (TEMED):

TEMED (Merck, Darmstadt) was used as accelerator of the polymerisation reaction

6.10. Preparation of SDS-PAGE gel:

The following proportion of the reagents described above were used:

15 mls of gel buffer (6.1)

13.5 mls of acrylamide solution (6.2)

1.5 mls of ammonium persulphate (6.8)

20 ul of TEMED (6.9)

6.11. Preparation of sample mixture for SDS-PAGE:

In the preparation of the samples to be loaded on SDS-PAGE, the following mixture of reagents was used.

	70% glycerol	40ul
	5% SDS	20ul
	50 ml phosphate buffer, pH 8.0	50ul
*	Beta-mercaptoethanol	5ul
	PMSF	5ul
	0.05% Bromophenol blue	10ul
	Sample	100ul
	Total volume	230ul

*If the sample was to be reduced and denatured, beta-mercaptoethanol was added in the quantitiy given above. The mixture (230 ul) was then boiled in a water bath for 2 minutes.

7.0 APPENDIX VII

Isoelectric focusing solutions and reagents.

Density gradient solutions for use with 1% Ampholine in the pH range 3.5-10:

All solutions were prepared according to the manufacturer's recommendations (LKB, Bromma, Sweden). The LKB 8100-1(110 ml) column was used for all the electrofocusing experiments.

(a) Dense gradient solution

Sucrose	27g
Distilled water	31ml
1% Ampholine	2ml
Sample	4ml
Total volume	54ml
Concentration of sucrose	50% (w/v)

(b) Light gradient solution

Sucrose	2.7g
Distilled water	48.3ml
1% Ampholine (R)	0.7ml
Sample	4.0ml
Total volume	54ml
Concentration of sucrose	5% (w/v)

(c) Electrode Solutions

(i) Cathode Solution

1M sodium hydroxide	2.5 ml
Distilled water	7.5 ml
Total volume	10.0 ml

(ii) Anode Solutions

1M phosphoric acid	4.0 ml
Distilled water	12.0 ml
Sucrose	15.0 g
Total volume	25.0 ml
Concentration of sucrose	60% (w/v)

7.2 Density gradient solutions for use with 1% Ampholine in the pH range 4-6

(a) Dense gradient solution

Sucrose	27g
Distilled water	34ml
1% Ampholine (R)	2ml
Sample	1ml
Total volume	54ml
Concentration of sucrose	50% (w/v)

(b) Light gradient solutions

Sucrose	2.7g
Distilled water	51.3ml

1% Ampholine (R)	0.7ml
Sample	1.0ml
Total volume	54ml
Concentration of sucrose	5% (w/v)

(c) Electrode Solutions

(i) Cathode Solution

1M sodium hydroxide	2.5 ml
Distilled water	7.5 ml
Total volume	10.0 ml

(ii) Anode Solution

1M phosphoric acid	14.0 ml
Distilled water	12.0 ml
Sucrose	15.0 g
Total volume	25.0 ml
Concentration of sucrose	60% (w/v)

APPENDIX VIII

8.0 Buffers and solutions used in the preparation of insoluble immunosorbents:

8.1 1mM hydrochloric acid solution for washing the freeze-dried cyanogen bromide activated Sepharose 4B

1mM hydrochloric acid solution was prepared by diluting 36% concentrated acid sp. gr. 1.18.

8.2. 0.1M sodium bicarbonate solution pH 8.3 with 0.5M sodium chloride (protein coupling buffer):

Sodium hydrogen carbonate	8.40g
Sodium chloride	29.22g

8.4g of sodium bicarbonate was dissolved in 200mls of distilled water, the pH was adjusted to 8.3 with 1M sodium hydroxide under magnetic stirring. 29.22g of sodium chloride was added and stirring continued until the salt had dissolved . The pH was checked again,and the volume made up to 1000 ml.

8.3. 1M Diethanolamine pH 9.0 (blocking agent):

Diethanolamne pure 105.14g was dissolved in 1000 ml:

8.4. 0.1M acetate buffer, pH 4, containing 0.5M sodium chloride (to wash away excess blocking agent):

Acetic acid	5.77g
Sodium acetate	8.2g
Sodium choride	29.22g

dissolved in 1000 mls distilled water.

APPENDIX IX

9.0. Buffers and solutions used in enzymeimmunoassays

9.1. Antigen coating buffer (carbonate buffer pH 9.6):

Sodium carbonate	1.59g
Sodium hydrogen carbonate	2.93g
Sodium azide	0.20g
Distilled water	1000 ml

The pH was adjusted to 9.6 using 1M NaOH.

9.2. Antiserum coating buffer (PBS, pH 7.4, with 2% PEG 6000):

9.2.1. 0.15M phosphate buffer:

di Sodium hydrogen phosphate (anhydrous)	21.3g
di Sodium dihydrogen phosphate	23.4g
Distilled water	1000 ml

To make 0.15M phosphate buffer, pH 7.4, 21.3g of disodium hydrogen phosphate was dissolved in 700ml of distilled water (solution 1), while 23.4g of sodium dihydrogen phosphate was dissolved in 1000 mls distilled water (solution 2). The pH was adjusted to 7.4 by mixing solution 2 and solution 1. The buffer was then transfered into volumetric flask and the volume made up to 1000 ml.

To make coating buffer, PBS was diluted 1/100 in distilled water, 2% w/v PEG 6000 and 0.1% sodium azide were added.

9.3. Buffer used in the dilution of antigen and antiserum:

Sodium chloride	30g
Calcium lactate	1g
Polyethylene glycol 6000	20g
Tween 80	0.5ml

Were dissolved in 1000 0.1 M borate buffer, pH 8.2.

9.4. Buffer used for the dilution of conjugate:

0.05M phosphate buffer, pH 8.0	1000ml
Potasium chloride	75g
Ethylenediaminetetraacetic acid, tetrasodium salt	1g
Tween 80	5ml
Benzoic acid	2.5g

The pH was adjusted to 7.5 using 4M NaOH. 5% pre-immune serum from the species in which the antiserum was prepared, was added.

9.5. Solution used for the washing of plates:

Phosphate buffered saline (PBS)	1000ml
Distilled water	9000ml
Tween 80	5ml

9.6. Buffer used in the preparation of substrate (0.05M) 3mls citrate/ammonium acetate buffer, pH 5.0):

3mls glacial acetic acid (17.5M) were diluted with 522 ml distilled water.

To 500ml of this 0.1M acetic acid solution, 10.5g citric acid were added.

The pH was adjusted to 5.0 using concentrated ammonia solution.

The buffer was transferred to a volumetric flask and the volume made up to 1000ml with distilled water.

9.7. The substrate solution sufficient for 1 microtitre plate:

0.05M citrate ammonium acetate buffer	10ml
20% Glucose solution	1ml
Horse radish peroxidase type II (100ug/ml)	0.1ml
2,2'-Azino-Di (3-Ethyl-Benzthiazoline sulfonic acid (ABTS), 50mg/ml 0.1ml	
Total volume	11.2ml

APPENDIX X

10.0 Buffers and solutions used in radioimmunoassays

10.1 TEN buffer pH 7.6:

20mM Tris hydrochloride	7.26g
1mM ethylenediaminetetraacetic disodium salt	1.116g
100 mM sodium chloride	17.64g
2mg/ml Bovine serum albumin (Fraction V)	6.0g

dissolved in 3000ml of distilled water.

10.2 Solutions and reagents used in the labelling of antigens:

(Hunter, 1979).

0.5M phosphate buffer, pH 7.5

Chloramine T	50mg/10ml
Sodium metabisulphite	240mg/80ml
Potassium iodide	1mg/ml

^{125}I was used in the labelling.

APPENDIX XI

11.0 Reagents and solutions used in the Folin Ciocalteu reaction for determination of protein contents:

11.1. Solution 1.

2% sodium carbonate in 0.1N sodium hydroxide.

Solution 2.

0.5% copper sulphate in 1% sodium tartrate was prepared freshly each day by mixing equal volume of double strength reagents.

Solution 3.

Alkaline copper solution was prepared by mixing 50 parts of solution 1 with one part of solution 2.

11.2. Phenol reagent

Commercial phenol reagent (Fisher Scientific Company, New York) was diluted in the ratio of 5 parts reagent to 4 parts of distilled water.

11.3. Bovine serum albumin (BSA)

BSA (No. A-4503, lot No. 107C-0307, Fraction V, Sigma Chemical Company, St. Louis, USA) was used to prepare the standard curve for determination of protein.

APPENDIX XII

Serum samples collected from slaughter houses

Total animals with bovine cysticercosis.....205

Total animals without bovine cysticercosis.....192

Animals with Fasciola only.....10

Animals with hydatid cysts only.....60

Number of sera from animals with other visible lesions
(hepatitis, calcified cysts, abscesses,
emphysema, peritonitis and oesophagostomiasis).....122

Number of serum samples collected.....397