"The use of a selected antigen in
enzyme immunoassay for the diagnosis of bovine
cysticercosis".

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the degree of Master of Science in the University
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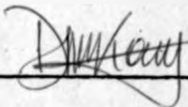

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DECLARATION
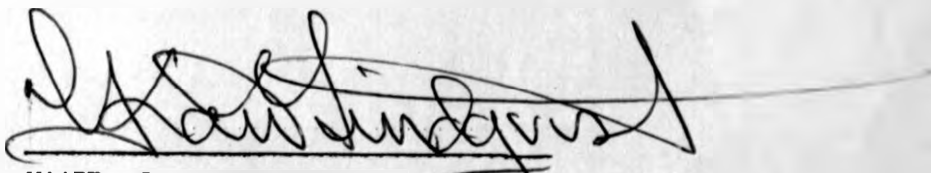
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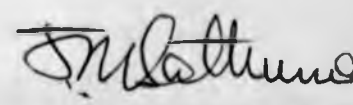
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DEDICATED TO MELICIANA, SELVIRIANI AND ALL THOSE WHO INSPIRED ME.

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SUMMARY

Human taeniasis due to T. saginata and
bovine cysticercosis caused by Taenia saginata
cysticercus (C. bovis) have a world wide distribu-
tion. Detailed information as to their impact on
human health and direct losses due to condemnation
of carcass meat or offal is difficult to obtain.
In Kenya alone, the annual loss has been estimated
to be K£20 million, and on a global basis the losses
are substantial. Apart from the economic con-
sequences ascribable to infected meat, cysticercosis
can be anticipated to become a barrier in the
expansion and development of international livestock
trade. With improved standards of hygiene and more
severe import restrictions on live cattle, only
cattle tested and found free of cysticercosis will
be allowed to be imported/exported. A reliable
serodiagnostic method may therefore be expected to
become necessary as part of the routine screening
of cattle for export or for slaughter. It is also
anticipated that a reliable serodiagnostic method
will be needed to single out infected cattle for
treatment if or when a suitable drug for bovine
cysticercosis has been developed. Furthermore,
specific immunological methods for cysticercosis may
serve to elucidate on the immune responses of cattle

in particular to parasitic infections. Finally, the theoretical possibility exists that serological tests may be needed to monitor or evaluate the effect of vaccination against cysticercosis.

Although standard meat inspection procedures are capable of detecting a portion of infected animals, they are generally regarded as inadequate for detecting low-level infections. Serological methods have not been able to offer an adequate alternative because they have generally lacked both sensitivity and specificity. It has been suggested that these deficiencies of the serological tests could be rectified by the use of antigen(s) specific for C. bovis and methods which possess high sensitivity. This study has attempted applying the concepts outlined above, in an effort to establish a sufficiently specific and sensitive serodiagnostic method for bovine cysticercosis.

The antigen selected for use in this study, antigen 13, was chosen from the 15 antigenic components of C. bovis as defined by crossed immunoelectrophoresis. An antiserum specific for this antigen was prepared and it was shown that antigen 13 could only be detected in T. saginata and its metacestode, T. hydatigena and its metacestode, C. cellulosae and T. hyenae/T. crocutae and its metacestode. It was absent in 19 other parasites tested. The molecular weight of antigen 13 was

estimated to be in the range of 220,000-250,000 daltons by a gel filtration technique. Although not entirely unique to C.bovis, the antigen was one among 3 antigens which are not shared with many other common parasites, and it was therefore considered suitable for further studies.

The method chosen for quantitation of anti-bodies to antigen 13 was an enzyme immunoassay which was deemed to possess the required sensitivity. A modification of this assay was introduced to allow the antibody response in terms of 50% inhibition titres on the basis of titration of one serum sample dilution and the use of a standard reference titra-tion curve.

The immunogenicity of antigen 13 was examined by immunisation of experimental animals with extracts of parasites which had been shown to possess antigen 13 in immunodiffusion and crossed immunoelectrophoresis tests. These animals gave considerably high 50% inhibition titres, while serum samples from animals immunised with extracts from parasites devoid of antigen 13 gave insig-nificant titres in the range of 3-14. These immunisation experiments, though remote from anti-genic stimulation which occurs in natural infections nevertheless showed that antigen 13 possessed satisfactory specificity and was also capable of

eliciting an excellent antibody response. Further-
more, the antibody assay system was found to be
sensitive and gave reproducible results.

The antibody response to antigen 13 was then
examined under conditions approximating those in
the field. Serum samples were obtained weekly
from calves infected artificially by oral dosing
with 200,000 eggs of T. saginata. The 50% inhibi-
tion titres to antigen 13 were determined over a
period of 4 months post infection. Distinctly
positive titres ($\geq$10) were observed 4-5 weeks
after infection and increased to 320 at 4 months
when titrations were terminated. Preinfection
serum samples and other known negative samples
consistently gave insignificant titres in the range
of 1-3.

The results obtained so far clearly indicated
that the inhibition enzyme immunoassay using anti-
gen 13, possessed the basic prerequisite required
for a serodiagnostic test for bovine cysticercosis.
Serum samples from 45 naturally infected and 85 non-
infected cattle were then examined with this method.
The results showed that the method was unsatisfactory
as a routine diagnostic test for cysticercosis in
naturally infected animals. Although the specificity
was very high (97.6%) a sensitivity of only 15.2%

is far too low, since even routine meat inspection procedures are more sensitive. It was apparent that the inhibition enzyme immunoassay using antigen 13 is only capable of effectively detecting massive infections.

The conclusions were further substantiated when serum samples from Wildebeest infected with C. crocutae, which shares antigen 13 with C. bovis were examined with the same method. Significant titres were only obtained with serum samples from animals having more than 100 cysts as determined by total body dissection.

In situations where sensitivity is unimportant and specificity becomes the determining factor, antigen 13 utilised in inhibition enzyme immuno-assay for diagnosis of bovine cysticercosis may be the method of choice. It is also possible that a more sensitive assay system can be developed which can still retain specificity through the use of antigen 13.

ACKNOWLEDGEMENTS

Sewage Treatment plant and Schools for their co-operation at the time samples were being collected.

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1. INTRODUCTION

1.   INTRODUCTION

Cysticercus bovis, the cause of bovine
cysticercosis is the intermediate stage of the tape-
worm Taenia saginata of man. Cattle are infected
by ingesting the eggs of Taenia saginata, and humans
are infected by eating raw or inadequately cooked
meat, containing cysts.

Taenia saginata is a common parasite of man
in many parts of the world. Bovine cysticercosis is
an important cattle disease because of the economic
losses incurred especially in developing countries.
It has been estimated that 80-100 million people are
infected with tapeworms including T.saginata (Mango,
1982). The incidence is higher in developing countries
where hygienic standards are low. Even in developed
countries, the incidence of taeniasis is increasing
(WHO memorandum 1976; Petrović et al., 1982). There
is a prevailing opinion that taeniasis is increasing
in many countries due to intensification of animal
production, development of meat industry by several
developing countries, increase in meat and live animal
trade, large scale intercountry migration of agri-
cultural and other workers, and ineffective sewage
disposal (WHO memorandum, 1976). At least 20 million
cattle are infected in Africa alone (Mann, 1978).
More than 10% of the cattle in Kenya, Tanzania, Congo,

Tunisia, Mali, Niger, Senegal and Djibouti are affected by bovine cysticercosis (Froyd, 1965; Dada and Belino, 1978; FAO-WHO-OIE Animal Health Yearbook, 1981). Incidence of up to 30% have been reported at some abattoirs in Uganda, Ethiopia, Zaire, Rwanda, Burundi, Swaziland, Ivory Coast, Central African Republic and Guinea (Bissau) (FAO-WHO-OIE Animal Health Yearbook, 1981). Of the total number of cattle slaughtered at the Kenya Meat Commission (KMC) abattoir at Athi River over a 10 year period, 22.8% had bovine cysticercosis (Gathuma, 1973). An increased incidence of bovine cysticercosis has been noted in several European countries (Pawloski, 1971).

The economic losses due to bovine cysticercosis are due to condemnation, downgrading and the cost of freezing retained carcasses. These losses are estimated at about K£20 million per year in Kenya and K£10 million per year in Botswana (Taylor, 1975, Grindle, 1978). According to WHO memorandum (1976), the effective way to reduce the incidence of bovine cysticercosis is by:-

- Education in proper sanitation and treatment of human reservoirs.
- Development of a reliable antemortem diagnosis of infected cattle.

-        Development of an efficient drug which will
kill the larval stages in cattle muscle without
lowering the quality of the meat.

Currently, the diagnosis of bovine cysticercosis
is carried out only during routine meat inspection,
after slaughter. However, the routine meat inspection
procedures are inadequate for the detection of
lightly infected carcasses (Walther and Koske, 1980).
Walther and Koske (1980) found that only 38.3% of
experimentally infected carcasses were detected by
classical meat inspection procedure, compared to 100%
found by extensive carcass dissection. They also
found that 56.7% were negative at the so-called pre-
dilection sites. There is an obvious practical limit
to the number of incisions which are permissible to
avoid gross mutilation of the carcass, which would lower
its marketability (Thornton and Gracey,1974). This
ineffectiveness of the meat inspection procedures in
diagnosing bovine cysticercosis and hence its control,
emphasises the need for more reliable diagnostic
methods. Ante-mortem serological tests are the methods
of choice for detection of infected animals. Effective
serodiagnosis of bovine cysticercosis is necessary to
detect antibodies or antigens in heavy, light or
chronic infections. This would allow for early diag-
nosis of the infection and the carrying out of
epidemiological investigations. When an efficient

drug has been developed to treat bovine cysticercosis, the antemortem diagnosis will be a pre-requisite if the treatment is to be useful and economical.

A good immunodiagnostic procedure is a result of the method applied and the quality of the antigen used. According to Kagan (1974), purification of antigenic components of whole parasites or their products may be the solution to the problem of specificity encountered in serological diagnosis. The purification and characterization of parasite antigens has been done by several workers (Dewhirst et al. 1967; Bout et al. 1974; Craig and Rickard, 1981). Partially purified antigens of C.bovis and T.saginata have been used to diagnose bovine cysticercosis (Gathuma, 1977; Geerts et al. 1981a). A number of workers have used enzyme immunoassays for the detection of circulating anti-bodies of C.bovis (Geerts et al. 1981b; Gallie and Sewell, 1983).

The elucidation of the immune response of the various antigens of C.bovis and T.saginata is impor-tant in this aspect. The objectives of this study were:-

i)   Define and partially characterise possible specific antigen(s).

ii)   Examine the cross-reactions of the specific antigen with other parasite antigens.

iii)   Attempt using the defined antigen in
        enzyme immunoassay to diagnose bovine
        cysticercosis.

iv)   Determine the presence of defined
        C. *bovis* antigen(s) in wildebeest
        cyst and its usefulness in the
        diagnosis of wildebeest cysticercosis.

2. LITERATURE REVIEW

The distribution of _Taenia_ species is limited by the availability of the host (final and intermediate) and may be influenced by climatic factors. This review covers the procedures for the diagnosis of _Cysticercus bovis_, purification and analysis of parasite antigens and the immune responses to the following cestodes:-

SPECIES	FINAL HOST	METACESTODE	INTER-MEDIATE HOST
Taenia saginata	Man	Cysticercus bovis (T. saginata cysticercus)	Cattle
Taenia solium	Man	C. cellulosae (T. solium cysticercus)	Pig
Taenia hydatigena	Dog	C. tenuicollis	Sheep and goats
Taenia ovis	Dog	C. ovis	Sheep
Taenia pisiformis	Dog	C. pisiformis	Rabbit
Taenia taeniaeformis	Cat	C. fasciolaris (Strobilocercus)	Mouse, rat
Taenia hyaenae	Brown hyena, Hunting dog	T. hyaenae cysticercus	Wild ruminants
Taenia crocutae	Spotted hyena	C. crocutae	Wild ruminants

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Taenia crocutae	Spotted hyena	C. crocutae	Wild ruminants.

2.1 IMMUNITY TO CESTODE INFECTION

Several theories have been advanced to account for the survival of a parasite in the host. While it may be conceded that the immune mechanisms may be inoperative or inefficient against helminths localized in the lumen of the gastrointestinal tract, most helminth parasites have complex life cycles which frequently involve migration and/or localisation in tissues. It is, therefore, to be expected that the migratory form(s) of the parasite are most likely to induce an immune response as well as being the most vulnerable to immune attack. Parasites apparently possess means to evade the host's immune mechanisms, either on the afferent or the efferent sides of the immune mechanism or both. The following proposals have been put forward to account for the phenomenon of evasion (Tizard, 1977; Kagan and Maddison, 1982).

i)   Shared antigens whereby the parasite syntheses host-like antigenic determinants.

ii)   Antigenic variation which is a method of escaping the host immunological attack.

iii)   Membrane turnover that could prevent host anti-bodies from reacting with tegumental antigens of the parasite.

iv)   Circulating soluble antigens that are detected

in serum. The soluble immunogens in the serum
are released by the parasite and may immuno-
suppress the host by acting directly on antigen-
reactive lymphocytes.

v)   "Trickle" infection occurs in some cases. The
less immunogenic of the soluble antigens
stimulate a weak response. The host is unable
to experience threshold infection and this
allows the persistence of the infection and
partial tolerance.

vi)   Acquisition of host antigens on the surface
of the parasite with a capacity for gradual
and repeated alteration.

The cestodes, like other parasites display some
of these phenomena (Kagan and Maddison, 1982).

2.1.1   CYSTICERCUS BOVIS

Resistance to this parasite can be acquired in
the course of natural infection, or by artificial
immunisation (Miller, 1931, 1934, 1935; Miller and
Kerr, 1932; Kerr, 1935). Passive immunisation
through hyperimmune serum and colostrum can also be
achieved (Miller 1931, 1934, 1935; Kerr, 1935).
Besides natural infection, studies have shown that
cattle infected orally with T.saginata eggs are
capable of developing protective immunity to challenge
infection (Penfold et al. 1936; Penfold and Penfold,

1937; Gallie and Sewell, 1972). T. saginata crude
extract incorporated in Freund's complete adjuvant
produced total immunity to challenge with Cysticercus
bovis (Gallie and Sewell, 1976). Different approaches
have been adapted to artificially immunize cattle
against cysticercosis by using artificially hatched
oncospheres (Wilkerhauser et al. 1974), viable or
irradiated eggs (Lucker and Vegors, 1965; Gallie and
Sewell, 1972, 1974), the excretions and secretions
of T. saginata oncospheres in culture (Kosminkov,
1973). The six-to eleven-day old calves which
received colostrum from cows vaccinated with in vitro
antigens of T. saginata during their last month of
pregnancy showed a high level of resistance to a
challenge infection with T. saginata eggs (Rickard
et al. 1977). These different methods of vaccination
resulted in a significant resistance to subsequent
challenge. The route and frequency of administration
of the vaccine are important. The intramuscular route
is more effective than the subcutaneous route
(Wilkerhauser et al. 1974), and repeated injections
were more efficient than the single one (Gallie and
Sewell, 1974). The strength of the antibody response
appears to be similar whether the antigens were from
adult tapeworm strobila or cysticerci (Gallie and
Sewell, 1976). In cattle, precipitating antibodies

against C.bovis reached detectable levels in
serum 8 weeks after experimental infection (Soulsby,
1963; Soule et al, 1971; Gallie and Sewell, 1972,
1974; Wilkerhauser et al. 1974). Rabbits produced
a humoral immune response against C.bovis in two
weeks after immunisation (Kamanga-Sollo, 1981;
Geerts et al. 1981a). Taenia saginata infections
in man produce haemagglutinating antibodies within
3 weeks (Machinika, 1974). Humans can establish
an immune response to a natural infection with
Taenia eggs (Flisser et al. 1979). C. bovis in
cattle can survive for 2 years (Dewhirst et al.
1963; Froyd, 1964; Urquhart and Brocklesby, 1965).
Under certain circumstances, C.bovis can survive for
a lifetime in the host (Urquhart, 1961).

2.1.2   CYCTICERCUS CELLULOSAE

Acquired resistance by natural infection or
artificial immunisation occurs in pigs infected with
C.cellulosae (Herbert and Oberg, 1974). In a study
of pig's immune response to infection with eggs of
T.solium, it was observed that the animals which
received a single large dose of eggs had more
cysticerci than those that received two small doses.
This indicates that large doses give better immunity
(Herbert and Oberg, 1974; Flisser et al. 1979).

C.cellulosae antigens are shared with other parasites including C.bovis antigens (Kamanga-Sollo, 1981).

2.1.3 TAENIA HYDATIGENA AND T. OVIS

Sheep were partially protected by immunising them with T.hydatigena eggs (Gemell et al. 1968, 1969). This was demonstrated by the reduced larval cyst count. Furthermore, Brundell et al.(1968) were able to confer a degree of immunity on lambs against a challenge infection with T. hydatigena by transfer of serum from immune sheep. Lambs were protected against infection with T.ovis eggs via maternal colostral antibodies from ewes which had been vaccinated before lambing or naturally exposed to infection. T. pisiformis and T. ovis were found to cross-protect animals from T.hydatigena infection. While T. hydatigena cross-protected animals against T. ovis infection, it did not do so with T. pisiformis (Gemmell, 1962, 1964, 1965, 1969, 1972). The cross-resistance was always less than that conferred by the homologous species. Complete resistance of sheep to challenge infection has been obtained with live, formalin fixed, or frozen embryoes of T. hydatigena and partial resistance with those of T.ovis (Gemmell, 1969). Because sheep kept on contaminated pastures were more resistant to challenge than those kept in

"sterile" zones, it is suggested that a natural immunisation process takes place (Gemmell, 1970, 1972).

2.1.4 TAENIA PISIFORMIS

Protein fractions of the ground-up worm material have been used in vaccination trials. Chromatographic fractionation of crude in vitro culture products of T. pisiformis failed to yield any fraction that produced protective immunity (Rickard and Katiyar, 1976). Lloyd and Soulsby (1974) suggested that IgG and IgA are the antibodies concerned with the resistance to challenge infection of both C. pisiformis and C. fasciolaris. Rabbits produced a humoral immune response against C. pisiformis in about 2 weeks after the first infection (Nemeth, 1971, 1972; Rickard and Outteridge, 1974). Complete resistance to challenge infection was induced in rabbits by introducing live embryoes by the sub-cutaneous (Heath, 1973) or intramuscular routes, (Gemmel, 1965).

2.1.5 TAENIA TAENIAFORMIS

The homogenate fractions of this worm provided protective immunity in vaccination trials in the cat. The polysaccharide fractions, however, were ineffec-

tive (Campbell, 1936, 1938). Using gel filtration
Kwa and Liew (1977) purified and characterised
crude homogenates and excretory products derived
from T.taeniaformis metacestodes maintained in vitro
culture. Live C.fasciolaris introduced into the
peritoneal cavity of mice (Rickard and Bell, 1971;
Musoke and Williams, 1976) as well as extracts
(Campbell, 1936; Kwa and Liew, 1978) of larvae induced
resistance to oral challenge with eggs of T.
taeniaformis. Studies of T.pisiformis infection in
rabbits (Heath, 1971) showed that immunity was con-
ferred mainly by antigenic fractions from oncos-
pheres or young cysticerci, containing six protein
bands in polyacrylamide gel electrophoresis. Four
were shared between the adult worm and cysticerci,
while the other two were unique to the cysticerci
(Heath, 1976). Injection of mice with larvae of
T.taeniaformis showed that IgG_{2a} was the most common
antibody during the first four weeks of infection
(Leid and Williams, 1974). Reagenic antibodies in
the rat and rabbit appeared in circulation within
2 to 3 weeks, then they declined. Thymectomy at
birth depressed immunity in some strains of mice and
this immunity could be restored by implantation of
the thymus tissue (Okamoto, 1968, 1970). Immunity
in such animals was also restored effectively with

bone marrow cell graft from similar strains of mice that had developed resistance to T. taeniaformis infection (Friedberg et al. 1969). In experimental cysticercosis of rats by C. fasciolaris, a delayed type of hypersensitivity has been demonstrated, but its participation in protection is not clear (Kwa and Liew, 1977). Fully developed cysticerci were resistant to immune attack while the early larval stages were susceptible (Musoke and Williams, 1975; Nemeth, 1970).

2.1.6 CYSTICERCUS CROCUTAE

This is the metacestode found in herbivores, particularly the wildebeest (Connochaetes taurinus). The adult worm's final host is the hyena. It is not yet clear whether the adult worm is Taenia crocutae or Taenia hyenae (Muchemi, 1982). There are reports of C. bovis in wild animals, especially in areas where humans have a high incidence of T.saginata infection (Stevenson et al. 1980). However, there is no evidence of adult wildlife tapeworms ever being found in man, despite the fact that wild animals are consumed by humans sometimes as raw meat. There is an antigenic relationship between C. bovis and C. crocutae (Kamanga-Sollo et al. 1982).

In the host-parasite system, there is a need to identify, isolate and characterize those moities

of the parasite which are responsible for the induc-
tion of host-parasite immune response (Laprange and
Capron, 1982). These moities of the parasite could
be used in very sensitive serodiagnostic tests like
enzyme immunoassay (EIA) and defined antigen sub-
strate spheres (DASS) to diagnose the tissue parasite
infections antemortem (Geerts et al. 1977).

2.2 PURIFICATION AND ANALYSIS OF ANTIGENS

The isolation, characterisation and purifica-
tion of the specific components of T. saginata and
C. bovis antigenic mosaic is important. The
evaluation of the antigens in a sensitive immuno-
diagnostic procedure is essential if specificity
is to be achieved in diagnosing the infection
(Soulsby, 1978; Kagan and Maddison, 1982). The
isolation and purification of antigens have been
done using various procedures by several workers;
chloroform gel fractionation (Fife and Kent, 1960),
isoelectric focusing (Melcher, 1943; Kagan and
Bargai, 1956; Reiber et al. 1961; Tarrant et al.
1965), ethanol fractionation (Seelman, 1960, 1961),
alcohol extraction (Fairly and Williams, 1923;
Mining and McFadzen, 1956), ion exchange and gel
filtration chromatography (Kent, 1963; D'Antonio
et al. 1960; Orihara,1967; Morris et al. 1968; Khan
and Meerovitch, 1970; Grossklaus and Walther, 1970;

Gathuma and Waiyaki, 1980), ammonium sulphate

precipitation (Urquhart et al. 1954) and affinity

chromatography (Geerts et al. 1979; Craig and

Rickard, 1981). Purified antigen fractions from

both adult and larval stages of T.saginata would

eliminate non-specific reactions that lower the

specificity of immunodiagnostic tests (Geerts et al.

1977, 1981a; Craig and Rickard, 1981). Host com-

ponents also contaminate most of the antigenic

preparations of parasites used in serodiagnosis

(Willms and Arcos, 1977). According to Enyenihi

(1974), purified antigens for detecting both human

and animal cysticercosis should be a mixture of

stage-specific antigens to avoid false negative

results. However, the purification of common antigens

to both stages of the parasite may overcome this

problem. Separation of T. saginata fractions that

could be used in the diagnosis of bovine cysticer-

cosis has been achieved by some workers (Gathuma,

1977; Geerts et al. 1981a). Geerts et al. (1981a),

using a fraction from T. saginata ("Fraction 10"),

were able to show group specificity to the taenid

family, a situation analogous to "Fraction 5" of E.

granulosus (Bout et al. 1974). It cannot, however,

be assumed that all antigens many of which are demon-

strated by serological methods (Kagan and Agosin,

1968), are automatically inducers of an immune res-
ponse in the host. The effectiveness of the purified
fractions of parasite antigenic moities in detecting
natural infections are yet to be thoroughly assessed
(Kagan and Maddison, 1982).

2.3   IMMUNODIAGNOSIS OF CESTODE INFECTIONS

During recent years significant progress has
been made in the development and improvement of
procedures for immunodiagnosis of parasitic diseases
(Fife, 1971; Kagan, 1974). The sensitivity and
specificity of an immunodiagnostic procedure depends
on the type of antigen employed and serological
technique used. In most cases, antigens have been
crude and procedures not standardised (Kagan, 1974).

2.3.1   IMMUNODIAGNOSIS OF BOVINE CYSTICERCOSIS

The diagnosis of bovine infection with C.bovis
using sera from experimental and naturally infected
animals has been done by several workers (Gathuma,
1977; Walther and Sanitz, 1979; Geerts et al. 1981b;
Gallie and Sewell, 1983). The presence of pre-
cipitating and haemagglutinating antibodies in bovine
sera infected with the growing metacestodes is a
favourable factor in immunodiagnosis. However, some

of these may not induce protective immune response, and if they do it may be minimal. (Gemmel and McNamara, 1972; Gallie and Sewell, 1974, 1976, 1983).

Some antigens of T. saginata are identical to those of C. bovis (Leikina et al. 1971). T. saginata extracts have been used as a source of antigen for the diagnosis of bovine cysticercosis by many workers (Froyd, 1963; Frick and Susse, 1970; Tailliez et al. 1976). Others have used T. crassiceps as a source of antigen (Geerts et al. 1981b). The use of purified antigens and very sensitive immunodiagnostic methods like enzyme linked immunosorbent assay (ELISA) have been suggested as the future ways of achieving sensitive and specific results for diagnosis of bovine cysti-cercosis (Enyenihi, 1974; Geerts et al. 1977; Voller et al. 1976; Voller et al. 1978). However, the results of ELISA on the proven cases of cysticercosis in conventially raised cattle showed poor sensitivity (Geerts et al. 1981b). Though, they used a crude antigen extract it can be said that the sensitivity of ELISA may not be as high as first thought in diagnosing parasitic infections (Ridley and Tosswill, 1982).

2.4 IMMUNODIAGNOSTIC METHODS APPLIED TO BOVINE CYSTICERCOSIS

The efficiency of classical meat inspection

procedure for diagnosis of C. bovis infection in abattoirs is low (Mann and Mann, 1947; Jespen and Roth, 1950; Gregoire et al. 1956; Dewhirst et al. 1967; De Vriers, 1973; Walther and Koske, 1980). The undetected cases may constitute a source of infection of epidemiological significance.

The review below summarises and evaluates the merits of individual serologic tests and an allergic test.

2.4.1   COMPLEMENT FIXATION TEST (CFT)

The antigen prepared from C. bovis was found to be non-specific and not sensitive enough by Frick and Susse (1970) and Lamina and Hein (1970). The same workers reported that a crude antigen prepared from T. saginata gave relatively better results than C. bovis in CFT. Soulsby (1963) and Omarov et al. (1973) found that when CFT was used to diagnose C. bovis infection, it was more sensitive at the time of cyst degeneration and calcification. This indicates that CFT could diagnose natural cysticercosis in cattle infected around six months of age; when there is a greater possibility of finding calcified cysts than in calves infected a few days after birth where viable cysts persist for a long period (Soulsby, 1963). In experimentally

infected animals, the highest titres were found
3 months after immunisation (Frick and Susse, 1970)
and became negative 6 months after immunisation
when CFT was used, though viable cysts were still
present (Urquhart, 1961).

2.4.2 IMMUNODIFUSION TEST

Although easy to perform, the gel diffusion
test has been found to be unreliable because of
frequent false positive results and cross-reactions
with other parasites (Trawinski, 1936, 1937; Dewhirst
et al. 1960; Maddison et al. 1961; Aksenova, 1973);
Gallie and Sewell, 1972). The method is, however,
able to detect heavily infected animals (Proctor
et al. 1966; Kagan, 1974). The inability of the
test to determine light infections and early antibody
response is a setback, when diagnosing infected
animals. Fractions of C. cellulosae gave positive
reactions when used to diagnose porcine cysticer-
cosis (Flisser et al. 1979).

2.4.3 INDIRECT HAEMAGGLUTINATION TEST (IHA)

The method has been used for a long time for
immunodiagnosis of parasitic diseases (Geerts
et al. 1977). Antigens prepared from the proglot-
tides of T. saginata or from the scolices and cyst

membrane of C. bovis have been used for the IHA
test by various workers (Dewhirst et al. 1967;
Alferova, 1969; Enyenihi, 1970; Frick and Susse,
1970; Gallie and Sewell, 1974; Tailliez et al. 1976;
Gathuma and Waiyaki, 1980).

Sheep erythrocytes have been most commonly
used as antigen carriers in IHA test for the diag-
nosis of bovine cysticercosis (Grossklaus and Walther,
1971). Bovine erythrocytes have also been used and
are said to eliminate some non-specific reaction
(Walther and Grossklaus, 1971). Tailliez et al.
(1976) are of the opinion that the use of glutaral-
dehyde as a coupling agent in the test provides a
more sensitive test than tannic acid. The haemag-
glutinating antibodies are usually detected in the
third week after infection with T. saginata eggs and
persist for at least 88 weeks (Alferova, 1969).
Omarov et al. (1973) found that the IHA test was
more sensitive during the height of a C. bovis
infection and not in the early or the late phases
of infection. The IHA test is a more sensitive test
than the CFT and intradermal test for the diagnosis
of bovine cysticercosis (Gallie and Sewell, 1974).
The IHA test has a setback of giving false positive
reactions. Attempts to eliminate these resulted in
loss of sensitivity (Mosina, 1965; Dewhirst et al.

1967; Martin, 1972). Gathuma and Waiyaki (1980),
using fraction called "FI" of T. saginata antigen
extract, reported high sensitivity and specificity
in IHA test as opposed to the use of a crude antigen.
The partial purification also reduced cross-reactions
with sera from animals infected with hydatid cysts.
Bianchi et al. (1968), found that a purified fraction
of .T. saginata gave the greatest haemagglutinating
activity compared to whole adult worm extract.

2.4.4   THE LATEX AGGLUTINATION TEST (LAT)

The most frequently used antigen of LAT has
been the extract of whole T. saginata, C. bovis
cyst and its scolices (Alferova et al. 1972; Filipov,
1971; Leikina et al. 1966; Sokolovskaya and Moskiv,
1967; Frick and Susse, 1970; Grossklaus and Walther,
1970, 1971; Kosminkov and Filipov, 1971; Martin,
1972). As in the case of IHA test, the LAT appeared
promising because of its high sensitivity. However,
the advantages of LAT are undermined by the presence
of large number of false positive reactions. Enyenihi
(1970) found a high percentage of false positive
reactions even with the use of antigen fractions.
The LAT gave more false positive reactions than the
IHA test. The incubation temperature is very crucial
for the sensitivity of the test (Grossklaus and

Walther, 1971). Omarov et al. (1973) concluded
that LAT gave best results during the early phase
and at the height of infection. Because of the
lack of specificity of the LAT Shekhovtov et al.
(1972) considered that this test is unsuitable for
field use.

2.4.5   THE INTRADERMAL TEST (ID TEST)

This is an allergic test. It is considered
a sensitive test, but is probably one of the least
specific. This is particularly true of the helmin-
thic infections (Fife, 1971). The ID test in the
diagnosis of bovine cysticercosis is a hypersensi-
tivity reaction (Bugyaki, 1961). Many homologous
and heterologous antigens have been employed by
various workers for the ID test on cattle for the
diagnosis of cysticercosis. There is no general
agreement between workers about the sensitivity of
a particular antigen (Bugyaki, 1961; Froyd, 1963;
Leikina et al. 1966; Frick and Susse, 1970; Bratanov
et al. 1974). The suitability of the sites for the
ID tests compared by Dewhirst et al. (1967) found
that an injection in the neck region gave a
sensitivity of 85%, whereas injection in the caudal
fold gave a sensitivity of 57.8%. The ID test gives
optimum reaction a few hours after injection. Schoop

and Lamina (1970) claimed that the assessment of
the reaction 24 hours after injection showed a
better correspondence with post-mortem findings
than the early hypersensitivity reaction. Eleven
months after infection, the test was negative,
although two of the 3 injected animals harboured
living cysts (Schoop and Lamina, 1970). The sens-
itivity of the ID test ranges from 65% - 100%
(Froyd, 1963). This test can be an attractive diag-
nostic method when a highly purified and specific
antigen is available (Geerts et al. 1977). By con-
trolling the nitrogen content and optimising effective
dilution of such a purified antigen, non-specific
reactions can be eliminated to a certain extent
(Kagan, 1968).

2.4.6   INDIRECT FLUORESCENT ANTIBODY TEST (IFA TEST)

The test is fairly sensitive for serodiag-
nostic procedures for a number of parasitic
infections (Geerts et al. 1977). It requires
only a small amount of serum. Cryostat sections
of C. bovis and T. saginata are the major antigens
used in various studies of IFA test (Calamel and
Soule, 1972; Euzeby and Dubra, 1970; Grossklaus and
Walther, 1971; Soule et al. 1971, 1972; Machnicka,

1973; Wilkerhauser et al. 1974). Cryostat sections gave more satisfactory results than extracts of C. bovis and T. saginata (Calamel and Soule, 1972). Variable and sometimes contradictory results are inherent in IFA test. By standardizing the antigen, all aspects of cross-reactions might be eliminated and the diagnostic serum titre determined (Geerts et al. 1977). Various serological tests for diagnosis of human cysticercosis were compared by Rydzewski et al. (1975). They found the IFA test promising for the diagnosis of bovine cysticercosis. Machnicka (1973, 1974), employing activated oncospheres as antigens in the diagnosis of bovine cysticercosis and taeniasis in man, observed specific immunofluorescence of cells of hexacanth embryoes of T. saginata. Gathuma et al. (1978) used hatched and activated oncospheres as antigen in IFA test and obtained high sensitivity and specificity with serum samples from cattle with cysticercosis. Gathuma and Waiyaki (1981), found the IFA test more sensitive and more specific than IHA test, using a crude T. saginata antigen. However, there were no significant differences between the sensitivities and specificities of the two tests when "F1" antigen was used in the IHA test.

2.4.7   IMMUNOELECTROPHORESIS (IE)

This method involves the principle of gel
electrophoresis and immunodiffusion. It was
developed by Grabar and Williams (1953). Geerts
et al. (1979) analysed the antigenic components
of T. saginata proglottides using IE method.
Twenty-two antigenic components were identified.
Most of these were of host origin and were not
specific for the cestodes. They observed that
serum from animals experimentally infected with
T. saginata eggs shows 3 specific precipitin lines
using this method. The major drawbacks of this
method are the false negative reactions in animals
with low infections (Geerts et al. 1980c).

2.4.7.1   COUNTER-IMMUNOELECTROPHORESIS (CIE)

This test has been used in the diagnosis of
both human taeniasis and experimental animal
cysticercosis (Betran and Gomez-Priogo, 1973). There
was good correlation between the clinical condition
of the cyst infection in the host and the number of
precipitin bands observed. Counter-immunoelectro-
phoresis in the diagnosis of bovine cysticercosis
as evaluated by Soulsby and Saquini (1974), was
found to be sensitive in detecting the infection.

This was based on the number and intensity of the
precipitin lines. False positive reactions with
sera from normal adult cattle were observed. This
technique was found to be about 10 times more
sensitive than immunodiffusion. In a review of CIE
in immunodiagnosis, Draper (1976) reported that the
test is less sensitive than the IFA test in the
diagnosis of protozoan and helminth infections. He
stated that the production of a more reactive and
specific antigen may improve the technique.

2.4.7.2   CROSSED IMMUNOELECTROPHORESIS

The original method was described by Laurell
(1965). The Clarke and Freeman's modification of
crossed-immunoelectrophoresis (1967) gave marked
improvements in the qualitive aspects of the method
(Axelsen et al. 1973). In one run several antigens
can be quantitated simultaneously. This can allow
identification of a number of proteins in serum
(Clarke and Freeman, 1968; Freeman and Smith, 1970;
Weeke, 1970). Crossed immunoelectrophoresis tech-
nique has been found to be superior to the classical
IE and CIE by providing a more powerful resolution
(Axelsen et al. 1973).

2.4.7.2.1 CROSSED IMMUNOELECTROPHORESIS WITH INTERMEDIATE GEL

This is a modification of the crossed-immuno-electrophoresis which was developed by Svendsen and Axelsen (1972). This technique has been used to characterize antigens and antibodies (Svendsen and Axelsen, 1972). The possibility of simultaneous demonstrations and characterization of free anti-bodies and antigens in serum makes it useful in infectious disease investigations (Axelsen et al. 1973). The method is also sensitive and suitable for the characterisation of complex antisera with weak precipitin lines (Axelsen et al. 1973).

2.4.8 RADIOIMMUNOASSAY (RIA)

This method was developed by Yalow and Berson (1960) and modified by Miles and Hales (1968). It is highly sensitive, being capable of detecting nanograms (10^{-9}g/ml) or picograms (10^{-12}g/ml) of antibodies in solution (Hunter, 1978). There are several modifications of RIA, the most used being the solid-phase RIA and radio-labelled antigen binding inhibition. RIA is more sensitive than any of the previously described methods (Ling and Overby, 1972; Wenzel et al. 1975). In the solid-phase RIA the radioactivity present is proportional to the

antibody present in the serum; while the radio-
labelled antigen binding inhibition RIA measures
the percentage of the antigen that is inhibited
to react with the antibody. The sensitivity of
this method is up to 0.5ng/ml (O'Reilly et al.
1975). In the diagnosis of bovine cysticercosis
not many workers have used RIA. Belozerow et al.
(1981) used RIA technique for diagnosing bovine
cysticercosis by utilising labelled bovine anti-
IgG and antigen from T. saginata ova or from
scolices of C. pisiformis. Kamanga-Sollo (1981)
used the method for experimentally infected cattle
to determine the antibody titres to "Antigen 11", a
highly immunogenic antigen of C. bovis.

2.4.9 ENZYME IMMUNOASSAY (EIA)

This method was developed separately by
Engvall and Perlmann (1971) and Van Weemen and
Schurs (1971). The enzyme immunoassay is useful for
the detection of antigenic determinants on molecules
in solution, on cell surfaces or for the investi-
gation of antibody specificities and affinities
(Engvall and Perlmann,1971). Enzyme immunoassay is
as sensitive as the corresponding radioimmunoassay
but has the advantage of more stable reagents and
fewer requirements for specialised equipment

(Voller et al. 1976, Voller et al. 1978). Arambulo
et al. (1978) described micro-ELISA technique for
serodiagnosis of human taeniasis. The results of
ELISA (Enzyme linked-immunosorbent assay) correlated
well with IHA test results when Walther and
Grossklaus (1981) used the test to diagnose bovine
cysticercosis. The IFA can detect circulating
antigens and antibodies in various infections. The
use of ELISA has'been applied by some workers to
diagnose bovine cysticercosis (Van Knapen et al.1979;
Craig and Rickard, 1980; Walther and Grossklaus,
1981; Geerts et al. 1981b; Gallie and Sewell, 1983).
Geerts et al. (1981b) found that it was most
difficult to detect infection in cattle harbouring
less than 100 cysts in ELISA. Among naturally
infected cattle, the sensitivity of ELISA was poor,
only 37.5 per cent. The use of T. crassiceps
antigen gave cross-reaction with serum from C. bovis
infected cattle and those from C. tenuicollis
infected sheep and Fasciola hepatica infected
cattle. The false positive reactions were at
4.3 per cent.

        Gallie and Sewell (1983) used ELISA to detect
the antibody titres in calves experimentally infected
with C. bovis. These were no detectable rise in
ELISA values in resistant calves after challenge
infection. In another study, Craig and Rickard

(1981) found that cross-reactions were significan-
tly reduced when a partially purified antigen of
T. saginata was used in ELISA. However, they
reported loss of sensitivity by using the partially
purified antigen. Duermeyer (1980) used enzyme
immunoassay for the detection of anti-hepatitis A
virus antibodies by the inhibition of the reaction
of enzyme and labelled anti-hepatitis A virus with
the antigen. Comparison of direct competition ELISA
and antibody inhibition ELISA shows that there are
instances where antibody inhibition method is pre-
ferred to direct competition ELISA (Rissing et al.
1980).

The simplicity of performance and availability
of specific antibodies make EIA an adaptable test
system, such as in automation. This could be used
in abattoirs for the detection of bovine cysticer-
cosis or porcine cysticercosis (Soulsby, 1978).

3.   MATERIALS AND METHODS

3.1   Antigens

3.1.1   Cysticercus bovis

3.1.1.1   Harvesting of C. bovis cysts

The C. bovis infected meat was collected from slaughterhouses in and around Nairobi and the Kenya Meat Commission abattoir, Athi River. The meat was collected in plastic bags, placed in an ice box and delivered to the laboratory. The cysts were carefully removed from the meat by dissection, on the same day. The cyst components and contents were separated into outer membrane, "outer fluid" and "inner membrane and scolex" of C. bovis (Kamanga-Sollo, 1981). The C. bovis antigen used was "inner membrane-scolex" (IMS) extract.

3.1.1.2   Preparation of the crude extract of inner membrane-scolex

1. Harvested the C. bovis cysts

2. Washed in physiological saline and then dry on blotting paper.

3. Added sodium azide to 0.01% and frozen at $-20^{\circ}C$ for storage until needed.

4. Ground with pestle and mortar frozen at $-20^{\circ}C$.

5. Homogenized in tissue grinder, placed in ice bath.

6. Sonicated at 300W for 5 minutes in ice bath.

7. This constituted a C. bovis crude extract.

8. Determined protein content.

3.1.2 T. SAGINATA

3.1.2.1 Collection of T. saginata

The T. saginata segments were collected from Kariobangi Sewage Works and from schools and helath centres in Kajiado District. The tapeworm segments collected from Kariobangi Sewage Works in Nairobi were retrieved just before entering the sewage treatment plant. They were placed in physiological saline containing 0.4% sodium azide and delivered to the laboratory. Tapeworm segments were also recovered from stools of the children after being treated with Yomesan[R] (Bayer, F.R. Germany) or Nocosamide (Cosmos, Nairobi, Kenya) followed by Epsom salt (E.T. Monks, Nairobi, Kenya). The recovered tapeworms were washed with distilled water and saline. The ploglottides were placed in plastic bottles containing physiological saline and delivered to the laboratory.

3.1.2.2   Preparation of T. saginata antigens

1. The segments were further washed with saline

2. Dried with a blotting paper

3. Added sodium azide to 0.4% and stored at -20°C until needed

4. Ground   with pestle and mortar

5. Homogenized with tissue grinder placed in ice-bath

6. Sonicated at 300W for 5 minutes in ice-bath

7. Centrifuged at 3000Xg for 15 minutes

8. Collected the supernatant

9. Determined protein content

This constituted the crude T. saginata extract. The protein content was determined by Lowry et al. (1951) method.

3.1.3   OTHER PARASITE ANTIGENS USED:

Other parasites were collected from places in and around Nairobi. Some parasite antigens were obtained from other workers working in various places in Kenya and outside. These included:-

		Host source
a)	_Fasciola_ species	Cattle
b)	_Moniezia_ species	Cattle, sheep, goat
c)	_Stilesia hepatica_	Sheep, goat
d)	_Paramphistomum_ species	Cattle, wildebeest
e)	_Avitellina_ species	Cattle
f)	_Oesophagostomum_ species	Goat, cattle
g)	_Cysticercus tenuicollis_	Sheep, goat
h)	Hydatid cyst fluid	Cattle
i)	_Trichuris_ species	Cattle
j)	_Ascaris suum_	Pig
k)	_Ascaridia galli_	Chicken
l)	_Trichostronglus_ species	Goat
m)	_Ancylostomum caninum_	Dog
n)	_Bunostomum_ species	Goat
o)	_Cooperia_ species	Goat
p)	_Setaria digitata_	Cattle
q)	_Haemonchus contortus_	Cattle,sheep,goat
r)	_Toxocara canis_	Dog
s)	_Taenia hydatigena_	Dog
t)	Hydatid cyst fluid	Human
u)	_Schistosoma japonicum_	Cattle
v)	_Cysticercus cellulosae_	Pig
W*	_Taenia hyenae_/_Taenia crocutae_	Hyena
X)	_Cysticercus crocutae_	Wildebeest
y)	_Spirometra pretoriensis_	Hyena, jackal

*These parasites are not distinguishable by morphological features. It is not yet clear how to distinguish the two parasites (Muchemi, 1982). The parasites were washed in water and saline and traces of sodium azide was added before storage at -20°C.

3.1.3.1   Harvesting of parasite cyst fluid antigen

C. tenuicollis and hydatid cyst fluid from sheep and goats were prepared in the following way:-

1. Collected the cyst from the carcass and transported in bottles with normal saline

2. Washed the cyst with water and normal saline

3. Bloted the cysts dry with blotting paper

4. Harvested the cyst fluid by puncturing the bladder into a collecting bottle

5. Concentrated by ultrafiltration using DIAFLO PM10 Ultrafiltration (Amicon, USA)

6. Added sodium azide to 0.1% and store at -20°C

3.1.3.2 Preparation of crude antigen extracts from the parasites

The following protocol was followed:

1. Harvested the parasite in saline with 0.2% sodium azide.

   ↓

2. Washed several times in saline

3. Ground ←——————— Stored at -20°C

   ↓

4. Homogenized in tissue blender placed in ice-bath

   ↓

5. Sonicated at 300W for 5 minutes

6. This constituted a   Lyophilized and
   crude saline extract   stored at -20°C or
                                4°C.

                                   ↓

                                Reconstitute to
                                required percentage
                                in saline

3.1.4 PREPARATION AND STORAGE OF ANTIGENS

All antigens apart from those used for immunisation of animals were stored at -20°C or 4°C with sodium azide as the preservative. Antigens once thawed were usually not frozen but stored at 4°C.

3.2 SEROLOGICAL PROCEDURES

3.2.1 Animals used

The antisera used in the study were prepared in the rabbits, goat and calves. The other sera were obtained from cattle, slaughtered at Kenya Meat Commission (KMC) abattoir, Athi River, and from wildebeests. Briefly, the procedures are described below.

3.2.1.1 Rabbits

Several New Zealand large white rabbits were bought from Veterinary Research Laboratories, Kabete and National Public Health Laboratories, Kenyatta National Hospital, Nairobi. The rabbits were treated for coccidiosis using Amprol (Soulsby Co., U.S A.). They were put in isolation and fed on concentrates (Rabbit pellets, Unga Ltd., Nairobi). They were provided with water ad libitum.

3.2.1.2 Goat

One goat of local breed was purchased from Dandora, Nairobi. It was hand-dressed with Delnav (Welcome, Nairobi) and ear tagged for identification. Regular deworming with Nilzan[R] (Wellcome, Nairobi) was carried out. The goat was housed in isolation and fed on hay, grass and concentrates (Ewe and lamb nuts, Unga Ltd., Nairobi). It was provided with water and salt lick (Maclick, Wellcome Ltd., Nairobi)

adlibitum.

3.2.1.3 Bovine serum samples

3.2.1.3.1 Samples from immunised animals

a)   Bovine serum samples were available in this
laboratory. They were obtained from calves that
were immunised with homogenate extracts of C.bovis,
T. saginata, hydatid cyst fluid and hydatid sand.
Parasite extracts were mixed with Freund's adjuvant
by sonication and used to immunise animals. Bleeding
and subsequent immunisation were done at two-week
intervals.

b)   Three Friesian calves which were 3 months
old from the University Farm, and considered to be
free from C. bovis infection were purchased. They
were dewormed and bled before immunisation. They
were dosed with 200,000 T. saginata eggs of 82%
viability. They were then bled at weekly interval
for several months. A pool of preimmune sera was
used as a negative control. Sera from another
calf (No.43/2) which had been raised in a taeniasis
free farm was used as negative control for the
standard reference curve in inhibition-enzyme immuno-
assay (INH-EIA).

3.2.1.3.2 Serum samples from conventionally
raised adult cattle

The serum samples were collected from Kenya
Meat Commission (KMC) abattoir, Athi River, during

process of slaughter. The animals slaughtered
came mainly from Narok and Kajiado Districts and
a few from other parts of Kenya. The positive and
negative serum samples were determined according
to the meat inspection procedures.

3.2.1.4   Wildebeest serum samples

These were provided by the Wildlife Research
Section, Veterinary Research Laboratories, Kabete
through the courtesy of Drs. L. Karstad and G. Muchemi.
The sera had been collected from experimentally
and from naturally infected wildebeests in the
Masai Mara Game Reserve, Narok District.

3.2.2   PRODUCTION OF ANTISERA

All animals were bled before immunisation.
The first injection consisted of the antigen with
complete Freund's adjuvant. The first injection
was done in superficial lymph nodes and intramus-
cularly according to the method described by Newbould
(1965). The subsequent boosters were done intra-
muscularly and subcutaneously. Bleeding was done
routinely at 2 weeks intervals and immunisation
boosters given 1 week after each bleeding (for a
period of about 20 months). Serum pools of different
bleedings were used for various serological tests.

3.2.3 PREPARATION OF MONOSPECIFIC ANTISERUM
TO AN ANTIGENIC COMPONENT OF C. BOVIS

The antiserum was produced in animals
(rabbits and goat) by immunising them with one of
the three precipitin lines obtained in immunodif-
fusion reaction between C. bovis extract and rabbit
No. 140 anti-C. bovis serum which had been absorbed
with various parasite extract insoluble immunosor-
bents. In the subsequent immunisation schedules,
T. saginata extract and absorbed rabbit No. 155 serum
precipitin lines were used. The precipitin line
choosen was one of the three antigens that were
specific for C. bovis and T. saginata and absent in
other common parasites that occur in cattle in East
Africa. The other two antigens are being investi-
gated in this department.

3.2.3.1 Preparation of precipitin lines of antigen 13
for the immunisation of rabbits

Twenty microscope slides were prepared as
described in 3.2.3.1. Immunodiffusion was allowed to
proceed overnight after precipitin lines were clearly
visible. The slides were then pressed and washed
as described in 3.4.1, but they were not stained.
The precipitin lines were cut out of the gel using
a clean razor blade (Gillete[R], England), as close
to the line as possible. Care was taken to avoid
contamination which could occur at this stage.

The agar strips containing the precipitin lines were then washed at 4°C with large volumes of PBS (pH 7.4), 3 times a day for 5 days. For every immunisation about 100 strips were used (20 micro-scope slides). At the time of immunisation, the PBS was sucked off and the strips sonicated in an ice-bath with Brausonic 1510 (Braun Messungen Ag, Germany), having an effect of 100W in second pulses until the agar was visibly dispensed. Freund's complete adjuvant was added and the mixture was sonicated as before, until the material became creamy. This was used for immunisation. The subsequent booster injections were prepared in the same way except that Freund's incomplete adjuvant was used.

3.2.4   ANTISERA USED

a)   Antisera prepared

		Antisera specifity	Preparation used
i)	Rabbit No. 183	anti-T. saginata (partially specific)	T.saginata/No.140
ii)	Rabbit No.169	anti-T.saginata (partially specific)	T.saginata/No.140 absorbed
iii)	Rabbit No.155	anti-antigen 13	T.saginata/No.169 absorbed
iv)	Rabbit No.175	anti-antigen 13	T.saginata/No.155 absorbed
v)	Rabbit No.D201	anti-antigen 13	T.saginata/No.155 absorbed
vi)	Rabbit No.401	anti-antigen 13	T.saginata/No.155 absorbed
vii)	Rabbit No. 402	anti-antigen 13	T.saginata/No.155 absorbed
viii)	Goat No.830	anti-antigen 13	T.saginata/No.155 absorbed.

b) Antisera jointly prepared with Dr. Kamanga-Sollo

		Antiserum	Preparation used
i)	Rabbit No.114	anti-T.saginata	T. saginata
ii)	Rabbit No.152	anti-C. crocutae	C. crocutae
iii)	Rabbit No. 150	anti-C. bovis (partially specific)	C. bovis/No.140

c) Other antisera used

		Antiserum	Preparation used
i)	Rabbit No.140	anti-C. bovis	C. bovis
ii)	Rabbit No.160	anti-antigen 11	C. bovis/No.140 absorbed
iii)	Rabbit No.199	anti-antigen 8	C. bovis/No.140 absorbed
iv)	Rabbit No.184	anti-Fasciola species	Fasciola species
v)	Rabbit No.166	anti-Moniezia species	Moniezia species
vi)	Calf No.846	anti-C. bovis	C. bovis

	Antiserum	Preparation used
vii) Calf No. 900	anti-_T.saginata_	_T. saginata_
viii) Calf No.28	anti-hydatid cyst fluid	Hydatid cyst fluid(bovine)
ix) Calf No.806	anti-hydatid sand	Hydatid sand (bovine)

These antisera were available in the Department of Public Health, Pharmacology and Toxicology, University of Nairobi.

x) Rabbit anti "Fraction No.10" antiserum produced by Geerts et al. (1981) of Antwerp, Belgium, was obtained through Prof. K.J. Lindqvist.

3.2.5 ABSORPTION OF ANTISERA WITH INSOLUBLE IMMUNOSORBENTS

Insoluble immunosorbents were used in this study to remove cross-reacting components of various parasites and host components used in immunisation.

The preparation was done by coupling a specific protein to Cyanogen Bromide activated Sepharose 4B (CNBr-Sepharose 4B-Pharmacia Fine Chemical, Uppsala Sweden).

3.2.5.1 Preparation of CNBr-Sepharose Immunosorbents

CNBr-Sepharose was bought from Pharmacia Fine

Chemical Co. (Uppsala, Sweden). The immunosorbent
was prepared according to the manufacturer's recom-
mendation. Briefly, the method was as follows:-

The required amount of CNBr-Sepharose was
washed with 1mM HCl in the ratio of 200 ml per gram
of dry sepharose. This was followed by washing with
copious amounts of distilled water. The protein to
be coupled was dissolved in coupling buffer (0.1M
sodium bicarbonate, pH 8.3,BHD, Poole, England).
The protein solution was mixed with the suspension
of the gel in a rotary mixer (Voss of Maldon, Essex,
England) for 2 hours at room temperature and then for
18 hours at 4^OC. The uncoupled protein, and the
remaining active groups were blocked by 1M ethano-
lamine at pH 9.0 for 2 hours at room temperature.
The blocking reagent was washed off using coupling
buffer, followed by 0.1M sodium acetate. The
was finally rinsed with coupling buffer and saline.
The efficiency of coupling was assessed by deter-
mining the uncoupled protein by the method of Lowry
et al. (1951). The sepharose gel conjugated with
the protein used was stored at 4^OC in 50% suspension
of saline with 0.1% sodium azide. The parasites
used to produce the immunosorbents were Fasciola
species, Moniezia species, T. saginata, Paramphistomum
species, Stilesia hepatica, Avitellina species and
whole rabbit and bovine serum.

3.2.5.3 Procedure of immunoabsorption of antisera
using insoluble immunosorbent

The absorption was done as follows: The anti-
serum was mixed with the immunosorbent and the
mixture was used in an end-to-end rotator at room
temperature (25°C) for 2 hours, then in the cold
for 18 hours at 4°C. The antiserum was separated
from the immunosorbent by filtering the suspension
through a sintered glass (G3-Pyrex, England). The
immunosorbents were regenerated by washing with 0.2M
Glycine/Hcl buffer; pH 2.8; followed by the coupling
buffer and saline. The immunosorbents were used
several times.

3.2.6   THE HARVESTING AND STORAGE OF ANTISERA

The preparation of antisera was done as described
by Campbell et al. (1970). Briefly, the method
involved the following procedure: Freshly drawn blood
was allowed to stand for several hours at room
temperature. The clot was retracted from the wall
of siliconised tubes using an applicator stick and
left overnight. The clot was removed and serum was
centrifuged at 3000 X g for 15 minutes to sediment
the red blood cells. Clear antisera were decanted
into universal bottles (United glass, England). For
preservation, 0.1% sodium azide was added to the

antisera,which were then stored at -20°C. After
bleeding, the antisera harvested were tested by
immunodiffusion using homologous and heterologous
antigens.

3.2.7 ISOLATION OF THE IgG FRACTION

An IgG fraction was prepared following the
method described by Fey et al. (1976). Slight modi-
fications were made as follows:

An equal volume of 100% saturated ammonium
sulphate solution was added slowly while stirring to
an equal volume of antiserum. The mixture was left
standing at room temperature (25°C) for 15 minutes
and centrifuged at 3000xg for 15 minutes. The
precipitate was washed twice with 35% saturated
ammonium sulphate, and dissolved in PBS. The immuno-
globulin fraction was dialysed against eluting
buffer containing 0.02% sodium azide for 18 hours
at 4°C. The dialysed fraction was then passed
through diethylaminoethyl cellulose (DEAE-cellulose,
Bio.rad, Laboratories, California, USA) column,
pre-equilibrated with 0.0175M phosphate buffer pH8.0.
The flow-through fraction was collected and con-
centrated by ultrafiltration using DIAFLO$^{(R)}$PM30
ultrafilter with a cut off point of 30,000 daltons.
Optical density (O.D.)of concentrated protein fraction
was read on the spectrophotometer (Beckman Model 25,

USA) at 280nm. The estimation of amount of IgG
in the solution was obtained using the formula
below (Givol and Hurwitz, 1969).

$$E^{1\%} = 13.5 \text{ at } 280nm.$$

3.2.8 CONJUGATION OF IgG AND HORSERADISH-
PEROXIDASE (HRPO)

The conjugation procedure adopted was the
method of Wilson and Nakane (1978) with the following
modifications.

IgG fraction of DEAE-cellulose chromatography
of the monospecific antiserum was used. Eighty per
cent efficiency of conjugation and final 1.1 molar
ratio of IgG/HRPO was assumed. Twenty milligrams of
HRPO (peroxidase Type IV, Sigma Chemicals, St. Louis,
Mo., USA) was dissolved in 4.5 ml of distilled
water. To it was added 0.5 ml of freshly prepared
0.15M sodium periodate solution (32 mg in 1 ml
water) under slow magnetic stirring for 30 minutes
at room temperature in the dark. The mixture turned
to a greenish brown colour. To it was added 0.1ml
ethyleneglycol, and continued stirring for 15 minutes
at room temperature. The pH should be about 4.5.
The solution was dialysed against 1mM cold sodium
acetate and several changes done. The pH is adjusted
to 9.0 using sodium carbonate (Na_2CO_3) under

stirring. Sixty milligrams of IgG solution is added slowly under stirring to the oxidised peroxidase (solution). The mixture was incubated in the dark at room temperature for 2 hours under stirring. The pH was then adjusted to 7.6 using dilute hydrochloric acid (HCL) and left overnight at 4^OC without stirring. Twenty milligrams of lysine were added and stirred for 15 minutes and left standing at 4^OC for 2 hours. The conjugated IgG solution was precipitated by adding equal volume of saturated ammonium sulphate solution. It was Centrifuged and the supernatant was saved to determine enzyme activity. Reconstitute the precipitate with a small volume of PBS (7 ml) and dialyse against PBS at 4^OC. Adjust the pH to 8.0 and filter with 0.45 u Millipore filter. Equal volume of glycerol was added (Koch-Light Laboratories Ltd., England). Stored at -20^OC or lyophilised and stored at 4^OC or -20^OC.

3.3   ANALYSIS OF ANTIGENS

3.3.1   Immunodiffusion

The microtechnique of Ouchterlouy double diffusion as described by Crowle (1973) was used with slight modifications. Briefly, the method was as follows:

1% (W/v) of purified Oxoid agar was dissolved in 100 ml of water and PBS (3:1) to obtain a medium for double diffusion. Sodium azide (0.1%) was added as a preservative. Four millilitres of molten agar was poured on the microscope glass slides (25mm) to give approximately a depth of 3mm. Using a gel-puncher (Gelman Instrument Co. Ann Arbor, Michigan), wells 4.0mm diameter and 5.0mm apart, were cut in a hexagonal pattern with a central well.

The agar in the wells was removed by suction. The central well was filled with 20μl of antiserum and each of the peripheral wells contained 20 μl of different antigens. This pattern was sometimes changed by placing the antigen in the centre well and specific antisera in peripheral wells. The slides were then placed in a humid chamber and diffusion allowed to proceed for 24 hours at room temperature (25°C). The precipitin bands were visible before and after staining. The bands were cut out as described in 3.2.3.1. or stained with Ponceau "S" or Commassie blue as described in 3.4.1.

3.4 CHARACTERISATION OF THE PURIFIED ANTIGEN

3.4.1 Crossed immunoelectrophoresis with intermediate gel (Svendsen and Axelsen, 1972)

The two-dimensional immunoelectrophoresis

was used for definition of antigen 13. In this
method an intermediate gel was interposed between
the first and the second dimensional gel of a
crossed immunoelectrophoresis. The technique was
described by Svendsen and Axelsen (1972) and was
done with slight modifications as follows:

One per cent (W/v) of Litex agarose (Litex,
type HSA, Denmark) in sodium barbital-calcium
lactate buffer pH 8.6, was used(Laurell, 1965).
The gel was 5mm thick on 80mm x 120mm plate. The
antigen well (in the 1st dimension) contained 30 ul
of antigen. The first dimension electrophoresis was
carried out for 60 minutes at 10v/cm. The second
dimension electrophoresis with an intermediate gel
was carried out on 50mm x 50mm glass plates using the
same 1% agarose mixed with 0.2ml of antiserum to make
a final volume of 2 ml. The intermediate gel made
up a volume of 1 ml. The electrophoresis was carried
out at 2 v/cm for 18 hours for the second dimension
at 4°C. The gel was pressed as described by Axelsen
et al. (1973) and washed in 3% (W/v) trisodium citrate
(Koch-Light Laboratories Ltd., England) of pH 8.5,
overnight. After washing, the plates were rinsed in
water, pressed, dried and stained with Commassie blue
(Sigma Chemicals, St. Louis, Mo. USA) for 40 minutes
and destained with Commassie destaining solution until
the background was clear. In some cases the inter-
mediate gel was blank while in other cases it contained

serum to be tested.

3.4.2 MOLECULAR WEIGHT DETERMINATION BY GEL

FILTRATION

An estimated molecular weight of antigen 13
was determined using four protein standards (Ferritin,
440,000 daltons; immunoglobulin G, 150,000 daltons;
bovine serum albumin 67,000 daltons; ovalbumin,
43,000 daltons, Pharmacia), chromatographed on
Sephadex G-200 column. The elution volume (Ve) at the
maximum optical density reading at 280nm was
recorded for each protein. This procedure was
repeated twice, and the average of the two values
for each protein was taken as the average Ve. Three
millilitres of filtered extract of T. saginata were
placed on the column (2.6 x 86 cm) and equilibrated
with PBS pH 7.4 with 0.1% sodium azide. The material
was eluted at a flow rate of 12 ml/hr at room
temperature. The elution volume of the antigen was
determined by detecting the highest $O.D._{280}$ in the
elute fractions using antibody sandwich ELISA
technique. The molecular weight determination was
calculated using the following formulae (Andrews,
1964, 1965):

$$Kav = (Ve - Vo)/(ve - (Vo)) \text{ and } R_{SA} = \frac{Ve \text{ serum Albumin}}{Ve \text{ of protein}}$$

where Vo is the elution volume for the blue dextran

200 (Pharmacia) and Vt is the total bed volume.
Using semilogarithimic graph paper, the Kav,RSA
and Ve for each protein standard was plotted on
the linear scale against the corresponding molecular
weight on a logarithimic scale.

3.5 PROCEDURE OF ENZYME IMMUNOASSAY

3.5.1 Inhibition Immunoassay (INH-EIA)

Disposable microtitration plates (Falcon[R],
USA) were coated with 100 ul/well T. saginata (120
ug/ml) extract in PBS. The plates were incubated
at 4°C overnight. Before use, the plate was washed
in a standard wash solution (0.015M PBS with 0.5%
Tween 80 and 1% chicken plasma plus 0.2% sodium
azide) 5 times at 5 minutes intervals. One hundred
micro-titres of a serum diluted in 0.5M NaCl/0.05M
borate buffer, pH 8.0 with 4% polyethylene glycol
(PEG) was added per well. The plate was incubated
overnight at room temperature. One hundred microlitre
of Goat No.830 anti-antigen 13-HRPO conjugate was added
without empting. The conjugate had been diluted to
one in forty in KCL/EDTA with 0.1% Tween 80 and
normal goat serum (5%). (This dilution had been
determined by checker-board titration of the antigen
and the conjugate). The plate was incubated for 1
hour at 37°C.   The washing procedure was

repeated. The substrate solution of 1 mg/ml OPD and 0.01 ml of 1% hydrogen peroxide diluted in 1 ml of 0.05M ammonium acetate/citrate buffer, pH 5.5 was added as 100 ul/well. The plate was incubated for 1 hour at room temperature. The reaction was stopped after 1 hour with 50 ul/well of 2N sulphuric acid. The plate was read at 490 nm, using a Micro ELISA[R] Minireader MR590 (Dynatech Instruments, USA).

3.5.1.1. Determination of 50% inhibition titres (50% IT)

The 50% IT were expressed as the reciprocal of that serum dilution which gave 50% inhibition in the test system. This titre was obtained in two ways: either by performing a complete titration curve using several dilutions of the test and control samples, or by using a single test sample dilution whose optical density (O.D.) readings were converted to percent inhibition and used to deter-mine the titre on the basis of a reference curve obtained with a known positive serum. In both methods, the O.D. readings were converted to percent inhibition using the formula:

$$\text{Percent inhibition} = \frac{(OD_z - OD_s)}{OD_z} \times 100$$

where OD_z represents the OD readings of wells representing zero percent inhibition, and OD_s

represents the O.D. reading given by a known dilution of test serum. The determinations of 50% IT based on standard reference curves are illustrated in Figs. 3.1 and 3.2. This inhibition method is based on the principle described by Duermeyer (1980).
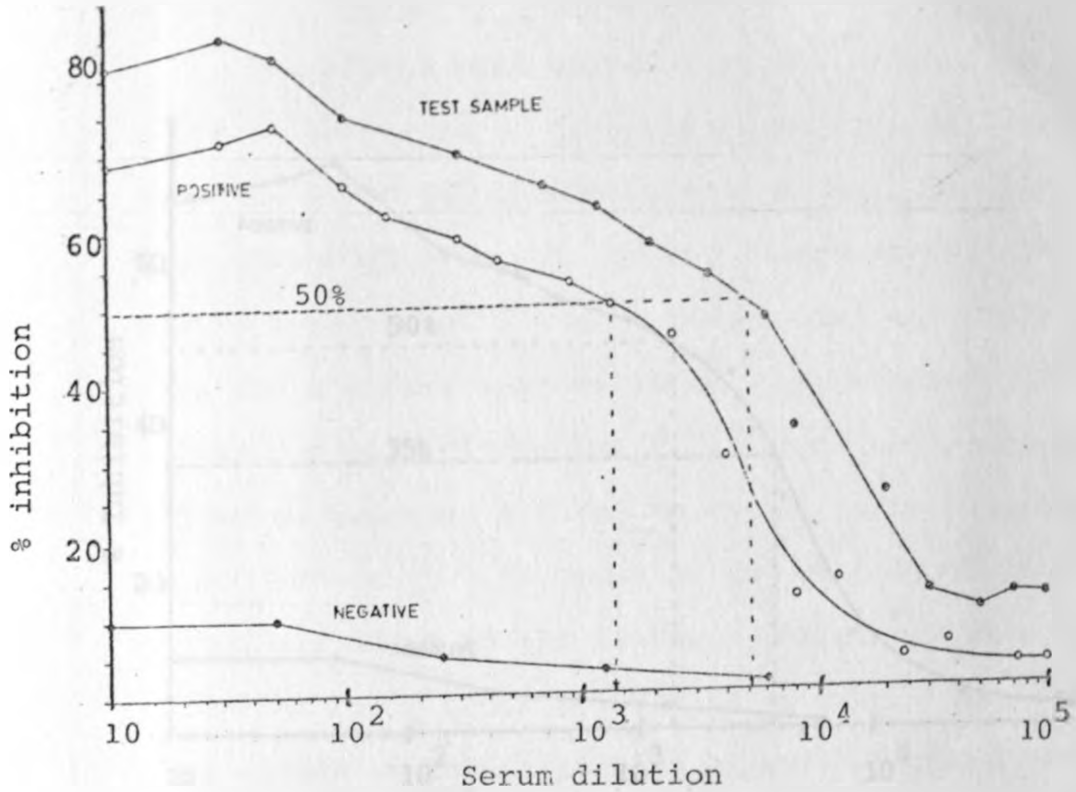
Fig. 3.1   Two complete titration curves
by which the 50% inhibition titre
using serial dilutions were directly
read off.
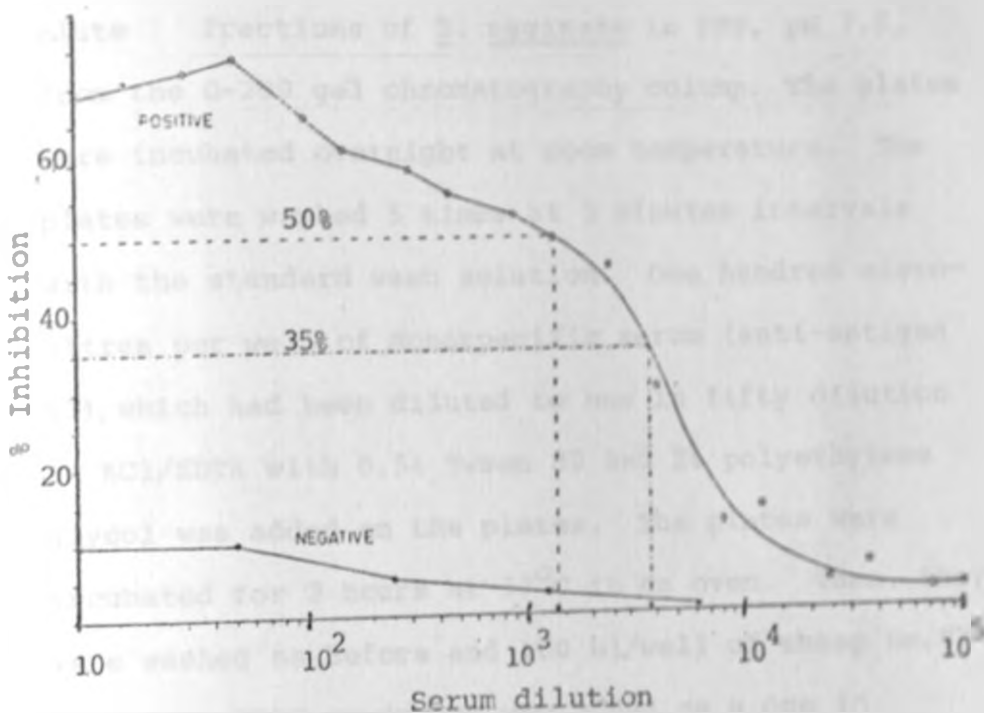
Fig. 3.2 The method of determining the 50%
inhibition titre in a single dilution of
the sample and the standard reference
positive serum.

The 50% IT (1350) of the reference serum
can be read directly from the curve. A
dilution of 1/100 of an unknown sample has
been calculated to give 35% inhibition. This
is utilized to get the dilution on the logar-
ithimic scale and the 50% inhibition titre
is calculated in the following way:

$$\frac{(1350)}{(3800)} \times 100 = 36 \text{ (50\% inhibition titre)}$$

3.5.2 ANTIBODY SANDWICH METHOD

The plates were coated with 100 ul/well of elute   fractions of $\underline{T}$. $\underline{saginata}$ in PBS, pH 7.5, from the G-200 gel chromatography column. The plates were incubated overnight at room temperature. The plates were washed 5 times at 5 minutes intervals with the standard wash solution. One hundred micro-litres per well of monospecific serum (anti-antigen 13), which had been diluted to one in fifty dilution in KCl/EDTA with 0.5% Tween 80 and 2% polyethylene glycol was added on the plates. The plates were incubated for 2 hours at $37^{O}C$ in an oven. Then, they were washed as before and 100 ul/well of sheep No.870 anti-goat-HRPO conjugate was added as a one in forty dilution in KCl/EDTA with 0.5% Tween 80. The incubation was carried out for 1 hour at $37^{O}C$. The washing using the standard washing solution was repeated as before. One hundred microlitres per well of substrate solution 0.01 ml of 1% hydrogen peroxide, 1mg/ml OPD in 1 ml of 0.05M ammonium acetate/ citrate buffer pH 5.5 was added. The plates were incubated for 1 hour at room temperature in the dark and 50 ul/well of 2N sulphuric acid added to stop the reaction. The optical density reading (O.D.) were taken at 490 nm using MicroELISA[R] Minireader MR590 (Dynatech Instruments, USA).

4.   RESULTS

4.   RESULTS

4.1   Antigenic constituents of C. bovis as

defined by crossed immunoelectrophoresis

analyses:

The presence of 15 antigenic components of
parasite origin was confirmed. On the basis of
this reference pattern, the antigen which was
selected for this study was identified as number
13 (Figs. 4.1a and 4.1b). The antigen 13 was
also present in T. saginata extract (Figs. 4.2a and
4.2b). The antiserum produced against antigen 13
was found to be specific and showed the presence
of this antigen in extracts of C. bovis (Fig. 4.3)
and T. saginata (Fig. 4.4).
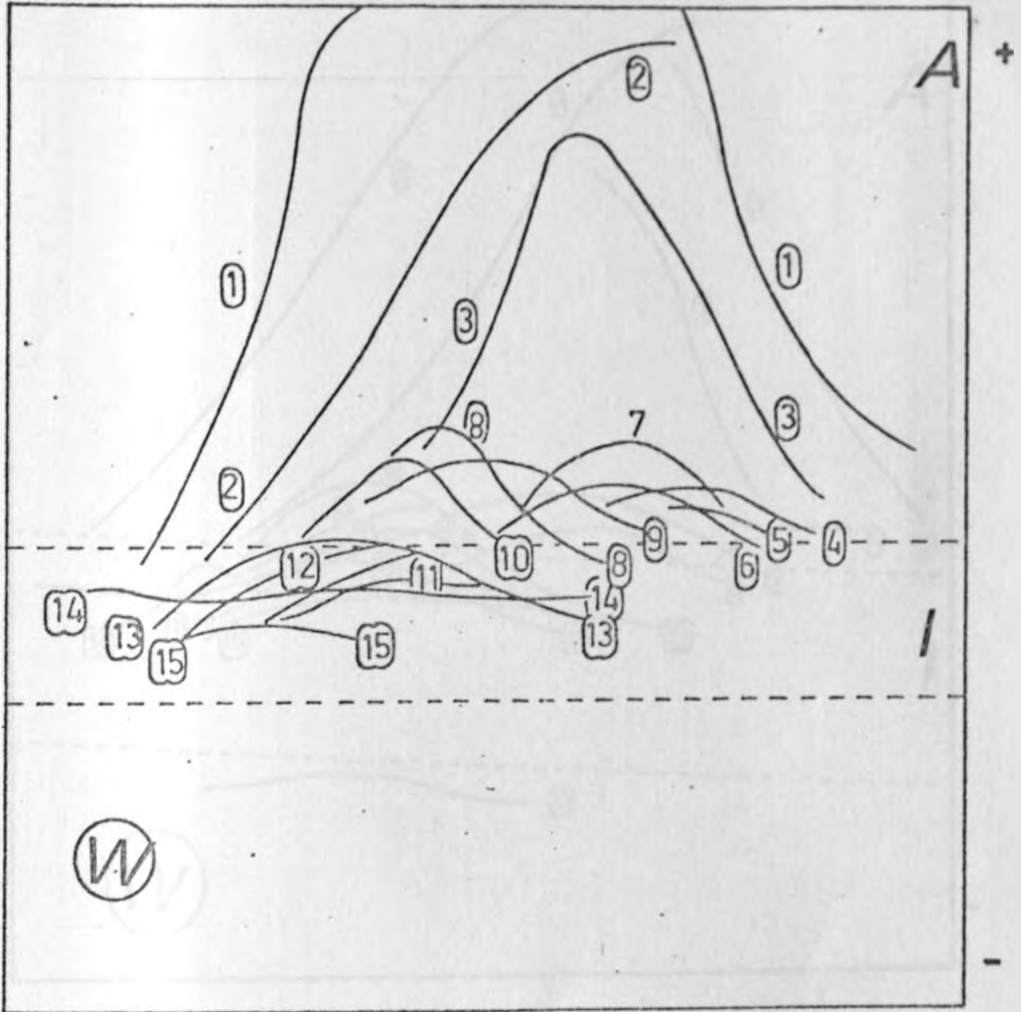
Fig. 4.1a.   Crossed immunoelectrophoresis
showing the antigenic components of C.bovis
in the established reference pattern.

W   -   C. bovis extract in agarose gel

I   -   Blank agarose gel

A   -   Rabbit No.140 anti C.bovis serum
        in agarose gel.
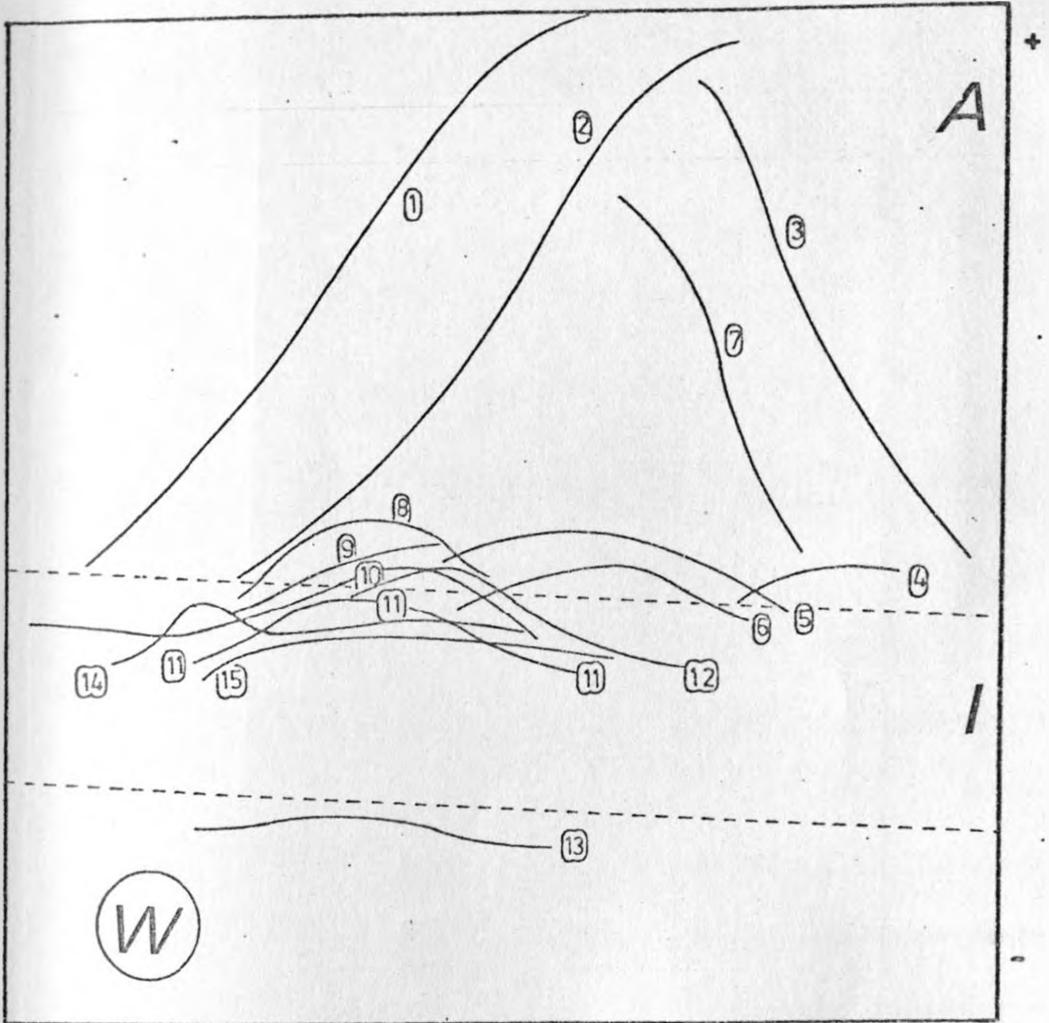
Fig. 4.1b.   Crossed immunoelectro-
phoresis with intermediate gel, showing
the position of antigen 13:

W   -   C. bovis extract in agarose gel

I   -   Rabbit No.155 anti-antigen 13
        serum in agarose gel

A   -   Rabbit No.140 anti C.bovis serum
        in agarose gel.
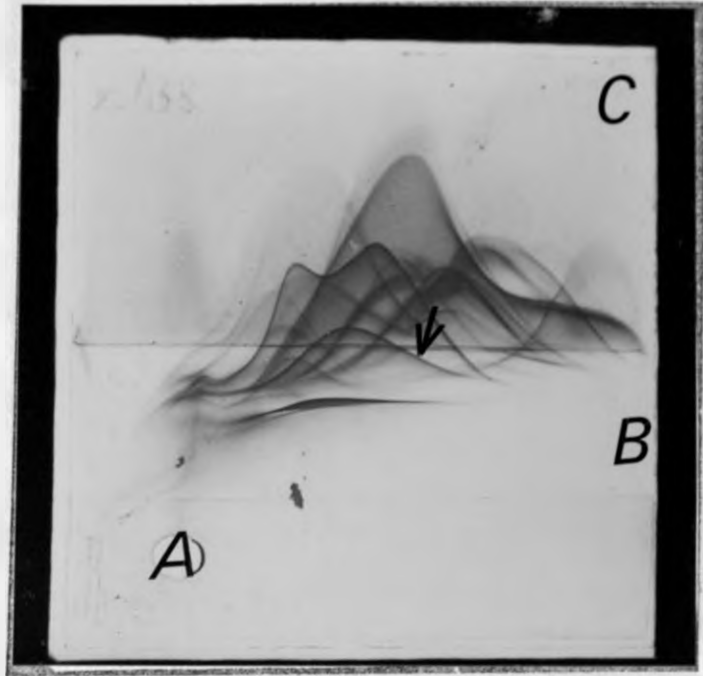
Fig. 4.2a   Crossed immunoelectrophoresis
             pattern with intermediate gel,
             showing the antigenic components
             of T. saginata.

             A - T. saginata extract in agarose
                   gel

             B - Blank agarose gel

             C - Rabbit No·114 anti-T.saginata
                   serum in agarose gel.

The·position of antigen 13 is

indicated by the arrow.
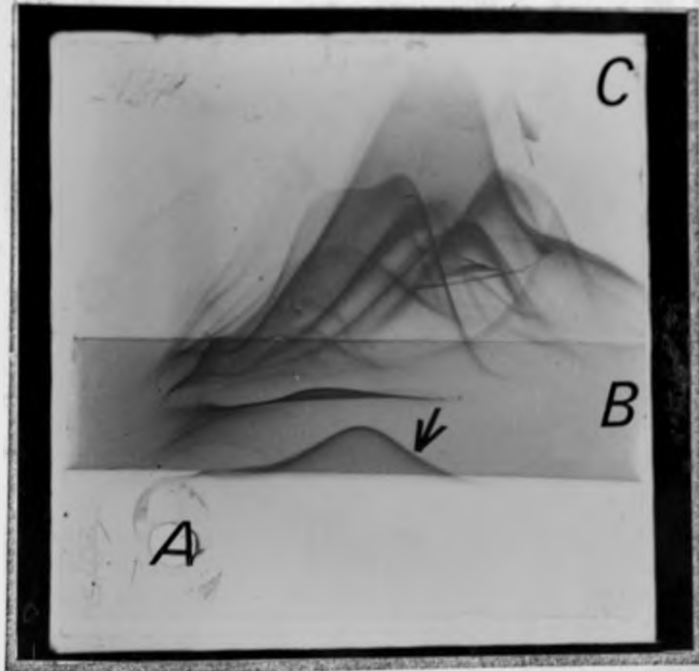
Fig.4.2b   Crossed immunoelectrophoresis with goat-
No.830 serum in the intermediate gel
showing the position of antigen 13,as one
of the antigenic components of T.
saginata:

A -   T. saginata extract in agarose
       gel

B -   Goat No.830 anti-antigen 13 in
       agarose gel

C -   Rabbit No.114 anti-T.saginata
       serum in agarose gel.

The arrow shows antigen 13 after being
separated from the other T. saginata
antigenic components.
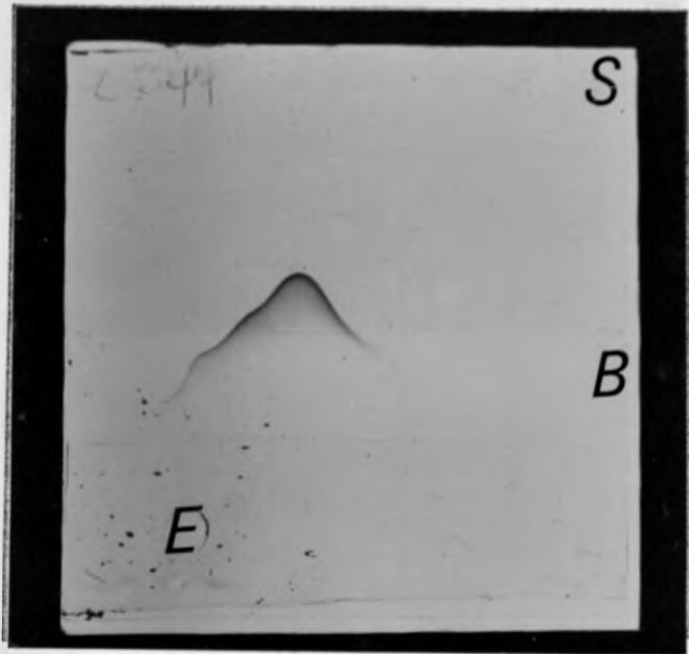
Fig.4.3   Crossed immunoelectrophoresis showing
           antigen 13 in C. bovis extract. The
           specificity of goat No.830 serum for
           antigen 13 is also evident.

E   -   C. bovis extract in agarose gel

B   -   Blank agarose gel

S   -   Goat No.830 anti-antigen 13 serum
          in agarose gel.
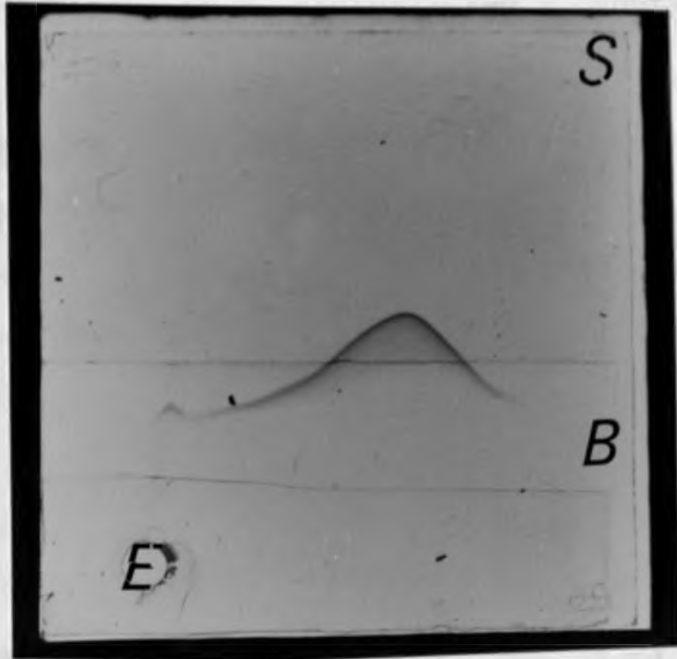
Fig.4.4   Crossed immunoelectrophoresis showing
          antigen 13 component in an extract of
          T. saginata. The specificity of Goat
          No. 830 serum for antigen 13 is also
          evident, since none of the many antigens
          of T. saginata has reacted.

          E _ T. saginata extract in agarose gel

          B _ Blank agarose gel

          S ⁻ Goat No.830 anti-antigen 13 serum in
              agarose gel.

4.2   COMPARISON OF ANTIGEN 13 WITH OTHER
ANTIGENS OF T. SAGINATA AND C. BOVIS

Using antisera against antigen 11 and
antigen 8 of C. bovis and antigen"F10"of T.saginata,
reactions of non-identity were obtained in immuno-
diffusion tests (Figs.4.5 and 4.6). The antiserum
against antigen 13 gave stronger precipitation
reactions with T. saginata extract than C. bovis
extract. This was in contrast to antigen 11 whose
antiserum gave stronger precipitin lines with
C. bovis extract than with T. saginata extract
indicating that the antigen is present in larger
amounts in T. saginata than in even concentrated
extracts of C. bovis.

69
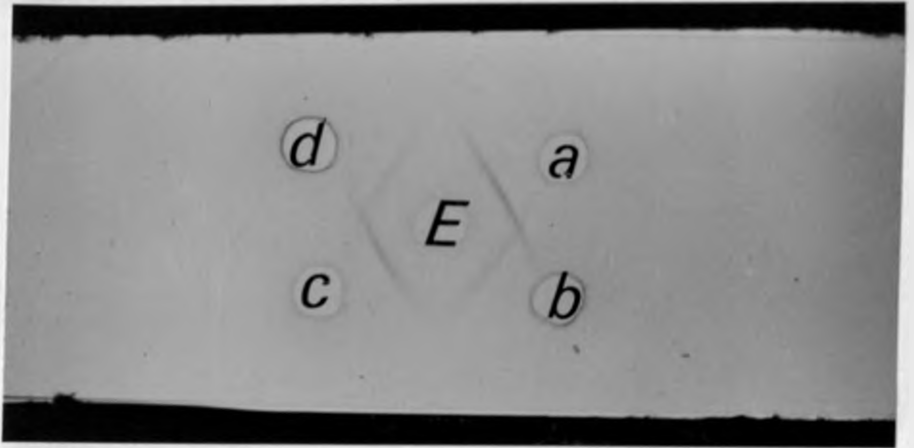
Fig. 4.5   Immunodiffusion experiment demonstrating
            reactions of non-identity between antigen
            13 and antigens 11 and 8 of C. bovis.
            The same results were obtained when a
            concentrated extract of C. bovis was used
            in the central well.

Well E -   T. saginata extract

Well a -   Goat No·830 anti-antigen 13 serum

Well b -   Rabbit No·199 anti-antigen 8 serum

Well c -   Goat No·830 anti-antigen 13 serum

Well d _   Rabbit No·160 anti-antigen 11 serum
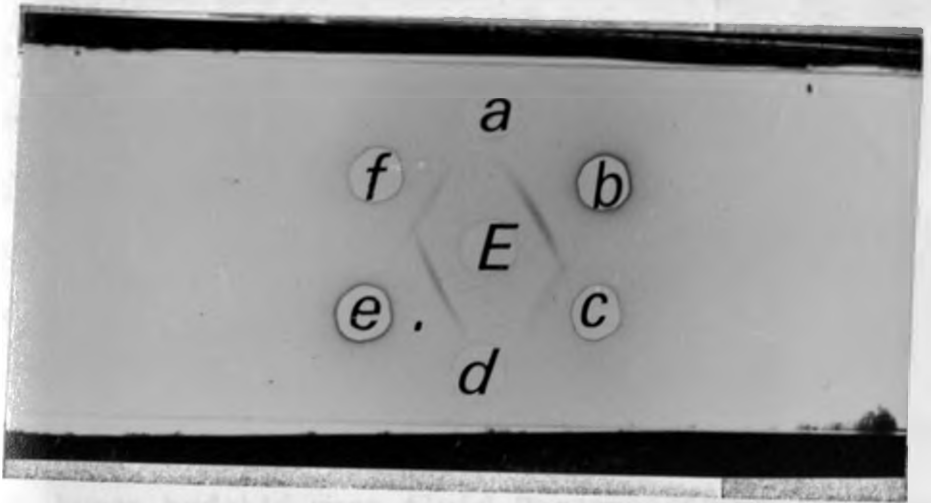
Fig. 4.6 Immunodiffusion experiment demonstrating
          a reaction of non-identity between antigen
          13 and the"F10"antigenic component of
          T. saginata.

Well E - T. saginata extract

Well a - Blank with saline

Well b - Goat No.830 anti-antigen 13 serum

Well c - Rabbit anti"F10"serum

Well d - Blank with saline

Well e - Goat No.830 anti-antigen 13 serum

Well f - Rabbit anti "F10" serum.

4.3 THE REACTIONS OF GOAT NO.830 ANTI-ANTIGEN 13 SERUM WITH VARIOUS PARASITE EXTRACTS

Only 4 of the 23 various parasite species examined possessed the common antigen 13 component. The antigen was present in T. saginata, C. bovis, T. hydatigena, C.tenuicollis, C. cellulosae, C. crocutae (Fig.4.7) and also T. hyenae/T.crocutae. Antigen 13 was not demonstrated in the following parasite extracts that were tested in immunodiffusion and crossed immunoelectrophoresis. Bovine hydatid cyst fluid, human hydatid cyst fluid Paramphistomum species, Fasciola species, Avitellina species, Moniezia species, Stilesia hepatica, Heamonchus contortus, Oesophagostomum species, Schistosoma species, Cooperia species, Spirometra pretoriensis, Ascaris suum, Ascaridia galli, Trichuris species, Trichostrongylus species, Setaria digitata, Ancylostomum caninum, Toxocara canis and Bunostomum species.

Fig. 4.7   Immunodiffusion test showing the presence
           of antigen 13 in taxonomically related
           parasite extracts.

Well A   Rabbit No. D201 anti-antigen 13 serum

Well 1   T. saginata extract

Well 2   C. bovis extract

Well 3   C. cellulosae extract

Well 4   C. crocutae extract

Well 5   C. tenuicollis extract

Well 6   T. hydatigena extract

4.4 ESTIMATION OF MOLECULAR WEIGHT OF ANTIGEN 13

The average elution volumes (Ve), Kav and R_{SA} values are given in Table I. The total bed volume (Vt) of the column was 456 ml and the void volume (Vo) determined by blue dextran was 144 ml.

Table I The Ve, Kav and RSA values of the protein standards and antigen 13

	Molecular weight	Ve(ml)	Kav	R_{SA}
Ovalalbumin	43,000	328	0.590	0.726
Bovine Serume Albumin (BSA)	67,000	238	0.301	-
Immunoglobulin G (IgG)	150,000	197	0.169	1.210
Ferritin	440,000	153	0.029	1.560
Antigen 13	220,00-250,000	176	0.103	1.35

From the plot on the semilogarithimic graph paper of the Ve, Kav and R_{SA}. values for the reference substances against the molecular weights of these proteins standards (Fig.4.8), the molecular weight of antigen 13 was estimated to be in the range of 220,000-250,000 daltons.
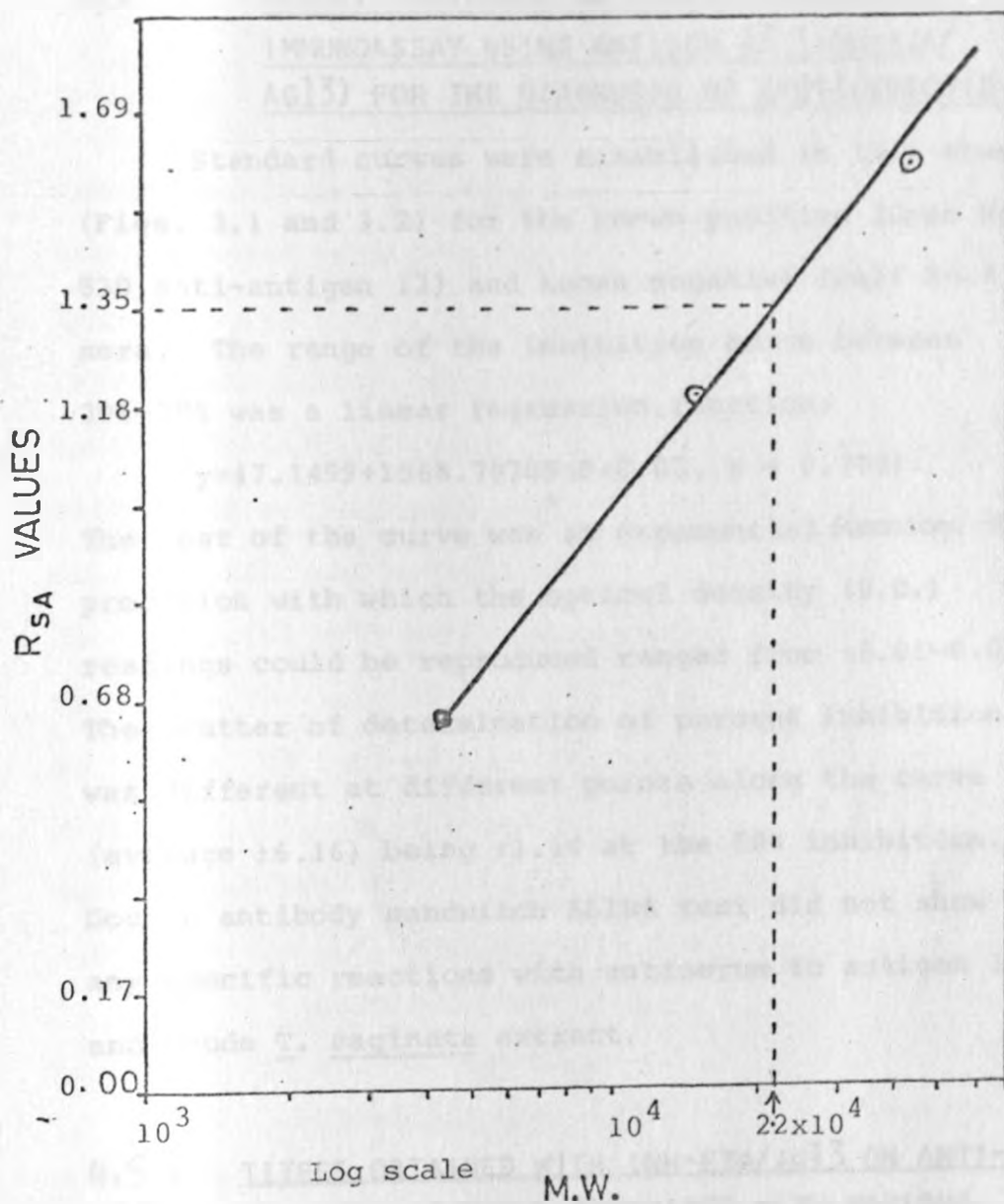
Fig. 4.8 Graph showing RSA values against standard molecular weight (M.W.) and the estimated M.W. of antigen 13 by G-200 gel filtration chromatography.

4.5 RESULTS OBTAINED IN INHIBITION-ENZYME
IMMUNOASSAY USING ANTIGEN 13 (INH-EIA/
AG13) FOR THE DIAGNOSIS OF CYSTICERCOSIS

Standard curves were established in this study
(Figs. 3.1 and 3.2) for the known positive (Goat No.
830 anti-antigen 13) and known negative (calf No. 43/2)
sera.   The range of the inhibition curve between
30%-70% was a linear regression function;

$$y=47.1499+1568.70709 (P<0.05, r = 0.708).$$

The rest of the curve was an exponential function. The
precision with which the optical density (O.D.)
readings could be reproduced ranged from ±0.01-0.08.
The scatter of determination of percent inhibition
was different at different points along the curve
(average ±6.16) being ±1.14 at the 50% inhibition.
Double antibody sandwitch ELISA test did not show
any specific reactions with antiserum to antigen 13
and crude T. saginata extract.

4.5.1 TITRES OBTAINED WITH INH-ETA/AG13 ON ANTI-
SERA FROM ANIMALS IMMUNISED WITH VARIOUS
PARASITE EXTRACTS

The test was done to determine whether anti-
bodies to antigen 13 could be detected by INH-EIA/Ag
13.   The antisera from animals immunised with para-
site extracts in Freund's adjuvant produced the
results given in Table II.

Table II   The 50% inhibition titres obtained
           with various antisera in INH-EIA/Ag13

Antiserum	50% Inhibition titre
Rabbit No.114 anti-T. saginata	8.0×10^6
Rabbit No.140 anti-C. bovis	2.2×10^5
Rabbit No.152 anti-C.crocutae	2.0×10^5
Calf No. 900 anti-T. saginata	1.2×10^3
Calf No. 846 anti_C. bovis	6.6×10^2
Calf No. 28 anti-hydatid cyst	12
Calf No. 806 anti-hydatid sand	10
Rabbit No.166 anti-Moniezia species	6
Rabbit No.184 anti-Fasciola species	3
Rabbit No.199 anti-antigen 8	14
Rabbit No.160 anti-antigen 11	8
Rabbit anti-"F10"	9
Rabbit No.169 anti-T.saginata (partially specific)	2.3×10^6
Rabbit No.183 anti-T. saginata (partially specific)	2.2×10^6
Rabbit No.150 anti-C. bovis (partially specific)	14

The rabbits routinely showed higher antibody titres than cattle despite extensive immunization schedules used in cattle, with similar or identical parasite extracts. Animals immunised with T.saginata extracts or antigens that were prevalent in T.saginata extracts after affinity chromatography gave higher

50% inhibition titres than in similar procedures
using C. bovis. Antigens 11, 8 and"F10"of C.bovis
and T.saginata which had been shown to lack common
antigenic determinants with antigen 13 showed as
expected insignificant 50% inhibition titres in
this test. Antisera from animals immunised with
parasite extracts which do not contain antigen 13
such as hydatid cyst, hydatid sand, Moniezia species
and Fasciola species, gave insignificant 50% inhibi-
tion titres. In contrast antisera from animals raised
against C. crocutae which has been shown to contain
antigen 13 gave high titres.

4.5.2 THE RESULTS OF INH-EIA/AG 13 FOR ANTIBODY TITRATIONS IN SERA FROM CATTLE EXPERIMENT- ALLY INFECTED WITH T.SAGINATA EGGS

The successful use of INH-EIA/Ag13 in animals
artificially immunised with parasite extracts indicated
promising results for detecting antibodies to antigen
13 in experimentally infected cattle. The calves
showed positive titres 4-5 weeks post-infection. In
the calves that were still alive 42 days post infection,high
50% inhibition titres were obtained (Fig.4.9). The
titres persisted up to 4 months when the monitoring
of the two calves was terminated. In the calf (No.
870) which died of unknown causes, the titres 5 weeks
post infection, were similar to those of the calves

which survived. A total of 25 cysts was counted
when total body dissection was done on the calf which
died.
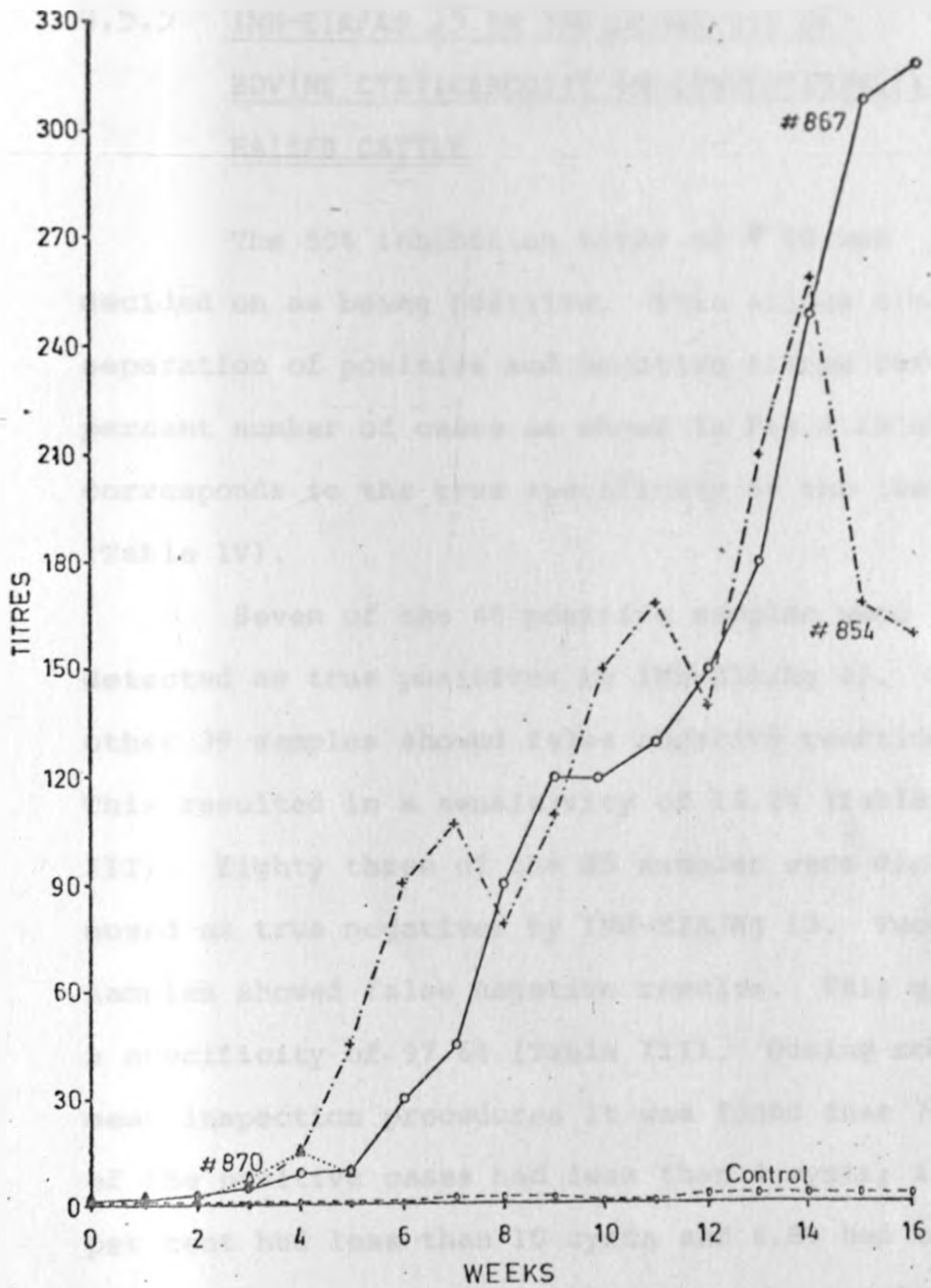
Fig. 4.9   The 50% inhibition titres obtained in
           INH-EIA/Ag13 found in experimentally
           infected calves in relation to time after
           infection with 200,000 T. saginata eggs
           (82% viability).

4.5.3 INH-EIA/AG 13 IN THE DIAGNOSIS OF BOVINE CYSTICERCOSIS IN CONVENTIONALLY RAISED CATTLE

The 50% inhibition titre of $\geq$ 10 was decided on as being positive. This allows clear separation of positive and negative titres for the percent number of cases as shown in Fig.4.10 and corresponds to the true specificity of the test (Table lV).

Seven of the 46 positive samples were detected as true positives by INH-EIA/Ag 13. The other 39 samples showed false negative reactions. This resulted in a sensitivity of 15.2% (Table III). Eighty three of the 85 samples were diagnosed as true negatives by INH-EIA/Ag 13. Two samples showed false negative results. This gives a specificity of 97.6% (Table III). During routine meat inspection procedures it was found that 74.3% of the positive cases had less than 4 cysts; 18.9 per cent had less than 10 cysts and 6.8% had ten or more cysts. Of the cattle that were sampled, 62.3% had multiple parasitic infections. The INH-EIA/Ag 13 showed high specificity in the diagnosis of C. bovis infection (Table V). It is apparent that the rate of detection of C.bovis infection using

antigen 13 became high with a high number of
cysts in the animal. Animals with more than 5
cysts had 42.9% chance of being detected.

Table III   Evaluation of the INH-EIA/Ag13 for
             diagnosis of bovine cysticercosis
             in conventionally raised cattle

		Postmortem diagnosis by meat inspection		
		+	−	Total
INH-EIA/Ag13	+	7	2	9
screening				
test	−	39	83	122
	Total	46	85	131

Sensitivity = 15.2% $(7/_{46})$

Specificity = 97.6% $(83/_{85})$

Table lV   Sensitivity and specificity in
            relation to titre levels of samples
            from conventionally raised cattle.

The 50% inhibition titre	Sensitivity	Specificity
Positive at ≥ 10	15.2%	97.6%
Positive at ≥ 5	20%	92%
Positive at ≥ 1	91%	8%

Table V   The relationship between routine meat

inspection results and the INH-EIA/Ag13

		Results in INH-EIA/Ag13	
	No. tested	No. positive	% Positive
C.bovis (>5 cysts)	7	3	42.9%
C.bovis (⁻5 cysts)	18	1	6.0%
C.bovis (varied number) with other parasites	20	2	10.0%
Hydatidosis	13	0	0
Fascioliasis	11	0	0
Lung emphysema	15	0	0
Liver cirrhosis and other lesions	7	0	0
Oesophagostomiasis	15	0	0
No lesions observed	24	2	8.3%
	130		
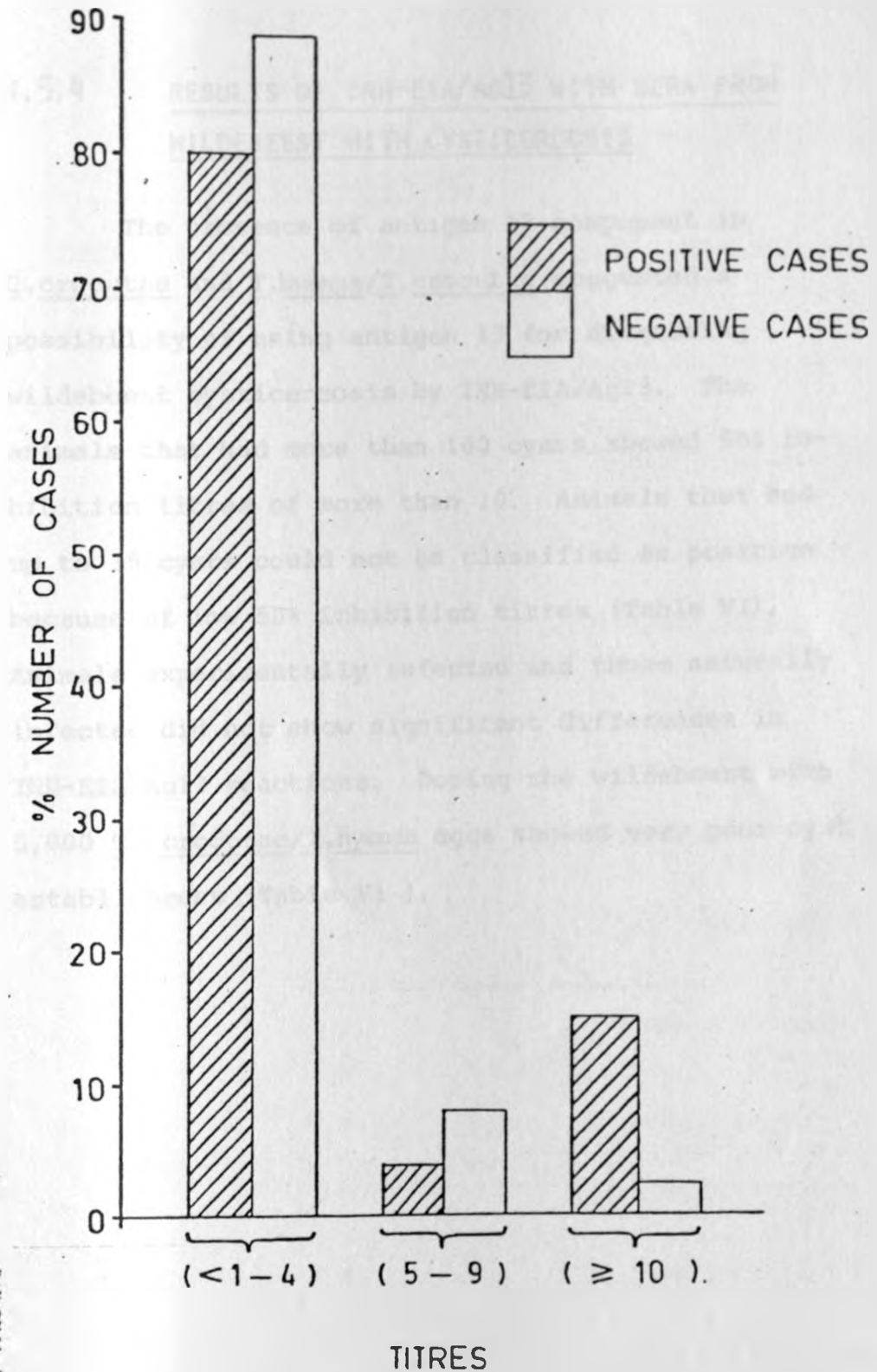
Fig. 4.10   Diagram showing the distribution of
50% inhibition titres obtained in
INH-EIA/Ag13 with sera from conven-
tionally raised cattle.

4.5.4 RESULTS OF INH-EIA/AG13 WITH SERA FROM WILDEBEEST WITH CYSTICERCOSIS

The presence of antigen 13 component in C.crocutae and T.hyenae/T.crocutae suggested a possibility of using antigen 13 for diagnosing wildebeest cysticercosis by INH-EIA/Ag13. The animals that had more than 100 cysts showed 50% inhibition titres of more than 10. Animals that had up to 35 cysts could not be classified as positive because of low 50% inhibition titres (Table VI), Animals experimentally infected and those naturally infected did not show significant differences in INH-EIA/Ag13 reactions. Dosing the wildebeest with 5,000 T. crocutae/T.hyenae eggs showed very poor cyst establishment (Table Vl).

Table Vl   The INH-EIA/Ag13 50% inhibition titres

in natural and experimental cysticercosis

of wildebeest.

Serial Number	50% inhibition titre	P.M.* findings and Remarks
W_4	40	150 cysts (Experimental infection)
W_3	19	140 cysts (Experimental infection)
W_H	17	100 cysts (Experimental infection)
W_G	3	35 cysts (Natural infection)
W_E	4	22 cysts (Natural infection)
W_D	7	22 cysts (Natural infection)
W_{45}	2	O cysts (Dosed with 5,000 eggs)
W_{46}	2	1 cyst (Dosed with 5,000 eggs)
W_{47}	2	2 cysts (Dosed with 5,000 eggs)
W_{48}	2	O cysts (Control)
W_2	2	O cysts (Control)
RO69	1	O cysts (Control)

* P.M. = Post-morterm.

5.   DISCUSSION

5. DISCUSSION

A number of serological methods have been
used in the antemortem diagnosis of bovine
cysticercosis. The enzyme linked immunosorbent
assay (ELISA) developed by Engvall and Perlmann
(1971) and Van Weemen and Schurs (1971) has been
shown to be a highly sensitive serological method
with a wide range of applications. It has also
been applied in the diagnosis of cysticercosis
(Van Knapen et al. 1979, Arambulo et al.1980;
Craig and Rickard, 1980; Walther and Grossklaus,
1981; Geerts et al. 1981b; Gallie and Sewell,
1983), but the results have been generally un-
satisfactory. Apart from the need for a sensitive
test, the use of a specific antigen is necessary.
The third prerequisite for a satisfactory immuno-
logical diagnostic method is that the antigen
chosen must regularly elicit an immune response in
the infected host, and preferably to low antigen
challenge, that is, low grade infections. Crude
parasite extracts have proved inadequate due to
frequent cross-reactions with other parasite
antigens. The use of these crude extracts is
particularly unsuitable for the immunodiagnosis
of a specific parasitic infection in areas where

multiple parasite infections frequently occur.
The use of antigen(s) with distinct specificity
has been recommended by several workers (Kagan,
1974; Enyenihi, 1974; Geerts et al. 1977, 1981a,
1981b) and Joint WHO/FAO/UNEP Expert Committee
(1976). Such antigens would eliminate or reduce
to an acceptable level non-specific reactions due
to cross-reactivity.

In this study, the antigen selected was shared
with parasites that do not infect cattle and are
taxonomically related to C. bovis and T. saginata.
This therefore, enabled it to be a suitable choice
as a candidate for immunodiagnosis of bovine cysti-
cercosis. Antigen 13 was one of the three such
antigens present in C. bovis, but showed no identity
with the other two antigens (antigen 11 and antigen
8). These other two antigens are being investi-
gated by other workers in this laboratory. Enyenihi
(1970) "purified" antigens of T. saginata which
gave cross-reactions with unrelated parasites such
as S. mansoni, E. granulosus, D. latum F.hepatica
and Neoascaris vitulorum. Antigen 13 was more
specific. It was estimated to have a molecular
weight ranging from 220,000-250,000 daltons by
G-200 gel filtration chromatography. The antigen

was found to be more abundant in the adult than larval stage. This allows the obtaining of antigen 13 in large quantities from T. saginata proglottides. The limited cross-reactivity of antigen 13 with various parasites made it suitable for use in diagnosis both experimental and natural cysticer-cosis.

Inhibition-enzyme immunoassay (INH-EIA) technique was utilised. This method gave better results than double antibody sandwitch-ELISA method when utilising crude T. saginata extracts. The method was reproducible with only moderate standard deviations of both optical density readings and percent inhibition. The inhibition enzyme immunoassay using antigen 13 (INH-EIA/Ag 13) was used in artificially immunised animals with parasite homogenates,experimentally infected calves and naturally infected cattle, giving various results.

In animals artificially immunised with parasite extracts, INH-EIA/Ag 13 showed the expected 50% inhibition titres that corresponded to the kind of parasite extracts used for the immunisation. Sera from animals that had been immunised with T. saginata extract or antigens that were specific to T.saginata gave very high 50% inhibition titres (1.2×10^3 - 8.0×10^6). Sera from animals immunised with C. bovis extract gave relatively lower titre (6.6×10^2 - 2.2×10^5) compared to T. saginata

extract immunised animals. The results here
confirms those shown in immunodiffusion test which
indicates that antigen 13 component is more in
T. saginata than C. bovis. In other animals
immunised with Fasciola species, Moniezia species,
hydatid cyst and hydatid sand, antibody titres
were insignificant (3-12). The same applied to
sera against antigen 11, 8 and "F 10" (8-14). Serum
from an animal that was immunised with C.crocutae
extract which has been shown to contain antigen 13
in immunodiffusion and crossed immunoelectro-
phoresis gave very high 50% inhibition titres
(2.0×10^6). The antiserum (No.150) to components
of C. bovis which did not include antigen 13 gave
very low 50% inhibition titres (14). Rabbits showed
much higher antibody titres than cattle,though
similar C. bovis or T. saginata extracts were used
for the immunisation. This has also been observed
by Kamanga-Sollo (1981). He indicated that cattle
are poor responders to antigens of C. bovis despite
extremely heavy immunisation schedules using ad-
juvants over a period of 18 months. He further
noted that calves immunised with the outer fibrous
membrane capsule did not respond to this component.
This may be not surprising as Slais (1970) had
suggested that the outer membrane is derived from

. the host tissue. The results in the artificially immunised animals show that antigen 13 is immuno-genic, specific and has high sensitivity in this assay system. This apparent specificity and sensitivity in INH-EIA/Ag 13 in artificially immunised animals indicated that it could be applied in the diagnosis of bovine cysticercosis.

The calves that were experimentally infected by oral administration of T. saginata eggs showed increasing antibody titres over a period of 4 months (Fig.4.9). A definite positive titre was obtained within 4-5 weeks post-infection. Walther and Grossklaus (1972) who investigated the heamag-glutinating response of calves and adult cattle infected with C. bovis, detected antibodies three weeks post-infection with T. saginata eggs. In adult cattle, Grossklaus and Walther (1970) using "active fractions" of T. saginata obtained positive titres in experimentally infected cattle 2-6 weeks by LAT method.

The presence of only 25 cysts in one of our experimentally infected calves which died 5 weeks post-infection, shows poor establishment of the cysts in that animal, despite a very high dose of eggs (200,000) with high viability (82%). However, the pattern of immune response before death looks similar to that of the other two calves. The monitoring of antibody levels in the

other two calves showed an increase over a 4
month period. At the time this investigation
was terminated, the degree of infection (i.e.
number of cysts) was unknown for the remaining
two experimental calves since they were kept alive
for further studies by other workers. From these
results it can be concluded that experimental bovine
cysticercosis can be diagnosed by the use of antigen
13 in the assay system employed in this study.

The possible usefulness of antigen 13 in the
diagnosis of bovine cysticercosis was then investi-
gated in natural infections. Unfortunately, the
low sensitivity (15.2%) precludes its use, although
the specificity was high (97.6%). Previous workers
who have used ELISA technique to diagnose bovine
cysticercosis reported as sensitivity of 37.5%
in naturally infected cattle, using crude T.
crassiceps extracts as the antigen (Geerts et al.
1981b). They also found cross-reactions with sera
from sheep infected with C. tenuicollis and F.
hepatica infected cattle. Of the animals that
were sampled which were conventionally raised, 62.3%
had multiple infections including fascioliasis and
hydatidosis. The specificity with which antigen
13 is able to distinguish these infections from
Taenia saginata cysticercosis is significant
especially in endemic areas. Kagan (1974) stated

that in his experience with sera collected at
slaughter houses in Kenya and Nigeria, the speci-
ficity of serological tests on sera from naturally
infected animals was poor. He rightly thought
that it could be due to cross-reactions with other
parasites in the endemic areas. In this study,
two of the 85 samples from cattle assumed to be
free from C. bovis at meat inspection showed positive
results in INH-EIA/Ag 13. This could be due to
the degeneration and disappearance of the cysts,
but persistence of antibodies to C. bovis. It
is also eminently clear that standard meat inspection
procedures cannot possibly detect low-grade
infection of C. bovis (Walther and Koske, 1980).

The low sensitivity (15.2%) in detecting
bovine cysticercosis in conventionally raised
animals by INH-EIA/Ag 13 indicates that it
cannot be used to diagnose natural cysticercosis,
despite high specificity (97.6%). In most infectious
conditions, critical to primary response are the
dose and quality of antigen (Sterzl et al. 1969).
A low level of antibodies may be due to insuf-
ficient stimulation, for example because of too
few cysts or because the antigen may be inacces-
sible to the host immune system. The waning of
antibodies in absence of restimulation by repeated
ingestion, may also play a role. Gallie and Sewell

(1974, 1983) showed that animals became resistant to challenge infection without increase in antibody titres. They found no detectable rise in ELISA values of resistant cattle following challenge. This could be due to an inability to detect protective antibodies by serological methods. Flisser et al. (1980) reported that more than 50% of the positive human cases of cysticercosis due to C. cellulosae appear not to be able to mount an immunological response to the parasite infection. They therefore suggested that large numbers of negative reactions found in sera from cysticercosis patients are not necessarily due to insensitive methodology, but due to insufficient humoral response of the host to the parasite.

Under natural conditions, bovine cysticercosis is, as a rule, a low grade infection. In this study, 74.3% of the infected animals had less than 4 cysts detected at meat inspection. Since INH-EIA/Ag 13 could detect only 42.9% of the infected animals with more than 5 cysts of C. bovis in the population that was sampled, the use of antigen 13 and/or the methodology are not adequate for routine diagnosis of bovine cysticercosis. However, it would be unrealistic to expect a few cysts to elicit sufficient immune response that

will be detected by the most sensitive serological methods.

The presence of antigen 13 in T. hyenae/T. crotutae and C. crocutae suggested the use of INH-EIA/Ag 13 in diagnosing Wildebeest cysticercosis. Again low sensitivity was found and definite positive titres were recorded in animals infected with more than 100 cysts. Geerts et al. (1981b) reported that it was difficult to detect infection in cattle with less than 100 cysts using ELISA technique. It is therefore clear that antigen 13 cannot be used effectively to diagnose infection in wildebeest, even though most wildebeests tend to be infected with numerous cysts (Karstad, personal communication, 1981).

The mechanism of immune response to established cysticerci and other aspects of host-parasite relationship need to be studied further. The important question of the identity of antigens which may confer protective immunity in C. bovis is not yet known. Carefully selected and defined antigen(s) and methodology could achieve a reliable antemortem diagnoses of cysticercosis. However, the sensitivity of the test using antigen 13 has to be considerably improved if the immunodiagnostic test is to be effective in the diagnosis of bovine cysticercosis.

6.　　CONCLUSIONS

6.   CONCLUSIONS

In this study, an attempt has been made at selecting an antigen that could be used in the ante-mortem diagnosis of cysticercosis. Based on the results obtained from the study, a number of observations and conclusions are made.

1. The selected antigen is specific for T. saginata and taxonomically closely related parasites and their larval stages. It is absent in most other common parasites. It was found to be abundant in adult than in larval stage of T. saginata. Its molecular weight was in the range of 220,000-250,000 daltons. It did not cross-react with other partially characterised antigens that were specific to C. bovis, and T. saginata and closely related parasites.

2. A modified method of ELISA was used. The standardisation of reagents and the method was done and found to be reproducible. The method allowed for more objective analysis of results.

3. In artificially immunised animals with parasite extracts, antigen 13 possessed satisfactory specificity and was found to be immunogenic. Rabbits were shown to respond more to the immune stimulation of antigen 13 than cattle.

4. In experimental bovine cysticercosis, the use of antigen 13 to diagnose the disease showed

positive titres 4-5 weeks post-infection. Again
the antigen was found to be immunogenic in cattle
experimentally infected with C. bovis. The
establishment of the cysts was found to be poor
in the animal on which a post-mortem (P.M.) exam-
nation was done, despite a high dose and viability
of the eggs used.

5.   Under natural conditions, massive infection of
cattle with C. bovis is rare. In Kenya, a high
number of cattle have multiple parasite infections.

6.   In naturally infected and non-infected adult
cattle the use of antigen 13 showed high specificity.
Cross-reactivity did not occur in common parasite
infections of cattle like hydatidosis, Fascioliasis
and Oesophagostomiasis.

7.   In the naturally infected cattle, the sensivity
of the test was very low. Because of this, antigen 13
cannot be used in the routine diagnosis of bovine
cysticercosis.

8.   The use of antigen 13 to diagnose wildebeest
cysticercosis showed low sensitivity, and like in
bovine cysticercosis, it is clear that antigen 13
cannot be used to diagnose natural infections of
wildebeest cysticercosis. The experimental
infections showed poor cyst establishment in
animals dosed with 5,000 eggs of T.hyenac/T.

crocutae.

9.   The antemortem diagnosis of bovine cysticer-
cosis needs both a specific and a sensitive test
to be effective in diagnosing natural infections.
Further studies are needed to develop a more
sensitive immunodiagnosis test which retains high
specificity. There is also a need to study the
immune response of cysticerci and other aspects
of host-parasite relationship. The important
question of the identity of antigens which may
confer protective immunity in _C_. _bovis_ infections
is another aspect that is not yet known and needs
to be studied.

7.   REFERENCES

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8.   APPENDICES

Appendix 1.1   Immunoassay results on sera from
conventionally raised cattle.   Positive cases by
meat inspection.

Serial Number		50% Inhibition titre
1	E47	< 1
2	B48	< 1
3	F59	1
4	N34	< 1
5	M1	1
6	M13	1
7	L2	1
8	B3	3
9	N27	3
10	K28	3
11	H28	2
12	A30	2
18	M47	2
19	M17	2
15	A34	< 1
16	N50	1
17	M49	2
18	C28	4
19	F53	3
20	M8	3
21	A27	2
22	M18	150

Serial Number		50% Inhibition titre
23	C39	3
24	C41	2
25	B26	2
28	F70	12
27	N14	25
28	M12	180
29	F18	2
30	F52	3
31	H27	1
32	F43	10
33	N4	1
34	E5	3
35	F16	3
36	A10	2
37	M43	1
38	F25	5
39	J14	2
40	M3	6
41	A19	2
42	F55	4
43	H7	4
44	A29	150
45	F71	4
46*	147	198

* Pool of some positive sera.

Appendix 1.2 Immunoassay on sera from conven-
tionally raised cattle. Negative cases by meat
inspection procedure

Serial Number		50% Inhibition titre
1	C46	4
2	J2	3
3	N11	3
4	C40	3
5	A28	2
6	E6	3
7	D8	2
8	N26	6
9	M15	3
10	A35	4
11	D6	8
12	C16	4
13	A36	4
14	N3	90
15	B5	3
16	N18	2
17	B10	8
18	A20	15
19	M27	3
20	L14	3
21	N16	2
22	N10	3
23	N9	1

Serial Number		50% Inhibition titre
24	C6	1
25	J18	4
26	A22	2
27	N21	2
28	M51	3
29	M44	1
30	M48	2
31	A26'	1
32	A37	1
33	C13	1
34	M46	< 1
35	C10	3
36	J12	1
37	D3	2
38	K19	< 1
39	C11	< 1
40	J10	2
41	N35	1
42	D13	< 1
43	A4	< 1
44	C8	< 1
45	M4	< 1
46	B46	1
47	C2	2
48	F1	5
49	F17	1
50	C42	1

Serial Number		50% Inhibition titre
51	M41	1
52	B47	4
53	L9	4
54	D18	5
55	B25	2
56	L4	4
57	L8	2
58	K40	2
59	B20	3
60	J1	3
61	C5	2
62	J21	2
63	M29	2
64	D14	1
65	M11	3
66	M13	3
67	A26	3
68	C9	3
69	N43	2
70	A6	3
71	D1	3
72	D7	3
73	C14	5
74	L15	3
75	N26	3
76	N28	2

Serial	Number	50% Inhibition titre
77	146	3
78	D4	6
79	M30	3
80	C18	3
81	J20	3
82	J13	3
83	N42	3
84	N48	3
85	J15	4

Appendix 2   Buffers and solutions used in

preparation of insoluble immunosorbent:

2.1   1mM HCl solution for washing and freeze

       dried cyanogen bromide activated Sepharose 4B.

       1 mM HCl solution was prepared by dilution

11.6M concentrated acid specific gravity 1.18.

2.2.   Protein coupling buffer

       NaHCO$_3$ 8.40g

       NaCl   29.22g

               8.4 g sodium carbonate was

dissolved in 2000 ml of distilled water, and pH

adjusted to 8.3 with 1M NaOH solution; 29.22g of

Nacl was added and stirring continued until the

salt had dissolved.   The pH was checked again.

The buffer was then transferred into volumetric

flask and the volume made to 1000 ml.

2.3   1M Diethanolamine buffer pH 9.0 (Blocking

       buffer):

       Diethanolamine pure 105.14 g/l buffer was

made into 1 molar solution.

2.4   0.1 M acetate buffer pH 4.0 containing

       0.5M Nacl to wash away excess blocking agent

       Acetic acid   5.77 ml
       Sodium acetate   8.20g
       Nacl   29.22g

               Dissolve in 1000 distilled water.

Appendix 3   Reagents and solutions used in

Lowry et al. (1951) method for

protein determination:

3.1   Solutions

Solution 1-2% sodium carbonate in 0.1M

NaOH

Solution 2-0.8% copper sulphate

$(CUSO_4.5H_2O)$ in 1% sodium tartanate

was prepared freshly each time the test

was done.

Solution 3 - Alkaline Copper sulphate

solution was prepared by mixing 50

parts of solution 1 to one part of

solution 2.

3.2   Phenol reagent

Commercial phenol reagent (Fischer

Scientific Co. NY.) was diluted in a ratio

of 5 parts phenol reagent to 4 parts of

distilled water (v/v).

3.3   Bovine serum albumin protein standards

BSA (Sigma Chemical Co. St. Louis, USA)

was used to prepare the standard curve

for determination of protein.

Appendix 4   Protein staining and destaining
              solutions:

4.1   Commassie Brilliant blue stain

        Commassie Brilliant blue 250R   10g

        Ethanol   900 ml

        Distilled water   900 ml

        Glacial acetic acid   200 ml

        The stain was filtered through Whatman No.1

filter paper to remove the insoluble material.

4.2.   Gel destaining solution

        Glacial acetic acid   200 ml

        Ethanol   900 ml

        Distilled water   900 ml

4.3   Ponceau "S" solution

        Ponceau "S"   2g

        1M acetic acid   1000 ml

        0.1M sodium acetate   1000 ml

Destaining solution for Ponceau "S"

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

Appendix 5   Buffers and solutions used in cross-
             immunoelectrophoresis (CIE) and
             immunodiffusion

5.1   Barbital - Calcium lactate buffer for CIE.

5.1.1 For electrophoresis chambers

       Sodium barbital (Na 5.5-diethylbarbiturate)
          105.1g.

       Barbital (5.5 - diethylbarbiturate) 16.6 g

       Calcium lactate   15.2 g

       This was made in 10 litres of distilled
water and adjusted to pH 8.5-8.6.

5.1.2 Preparation of Agarose

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

5.2   PBS for preparation of agar gel for
       immunodiffusion and other uses:

5.2.1 PBS (0.15M, pH 7.4)

       Di-sodium hydrogen phosphate (anhydrous)
21.2g. This was dissolved in about 800 ml of
distilled water, the pH was adjusted to 7.4 by
using dilute Hcl acid (1N HCl), the volume was
then filled to 1000 ml in the volumetric flask.

5.2.2 PBS, pH 7.4

1 volume of 0.15M phosphate buffer pH 7.4 was added to 9 volumes of saline (0.9% sodium chloride in distilled water).

Appendix 6   Agar and Agarose used in immuno-
               diffusion and CIE

6.1   1% Agar in PBS pH 7.4 for immunodiffusion

       Purified Oxoid agar   2g

       PBS   50 ml

       Distilled water   150 ml

       Sodium azide (NaN$_3$)   0.02g

            0.1% (w/v) sodium azide was added

       as a preservative to prevent microbial

       growth on the agar during immunodiffusion.

6.2   1% Agarose in sodium-barbital: Calcium

       lactate buffer with Triton X-100 for CIE

       Litex agarose (type HSA)   2g

       Na-barbital/ Ca-lactate buffer   50 ml

       Distilled water   150 ml

       Triton X-100 (p-Isooctylphenoxpoly-
            ethoxyethanol)   100 ul

Appendix 7   Buffers, diluents and solutions

used in enzyme immunoassay:

7.1   Standard washing solution

Phosphate buffered saline pH 7.4   1000 ml

Distilled water   9000 ml

Tween 80   5 ml

Chicken plasma   50 ml

Sodium azide   1 g

7.2   Serum samples diluent-0.5M NaCl/0.05M
       borate buffer pH 8.0

To 800 ml of distilled water add,

Sodium chloride   29.22 g

borate buffer   3.1 g

PEG   40   g

Adjust pH 8.0 with concentrated 4M NaOH.

Fill up to 1000 ml with distilled water.

7.3.   KCl/EDTA diluent for conjugate

0.05M phosphate buffer pH 8.0   1000 ml

KCl   75 g

EDTA   1g

Benzoic acid   2.5g

Tween 80   5 ml

Adjust the pH to 7.5 with 4M NaOH

solution. Add chicken plasma or normal serum to

the required percentage. If the diluent was to

be used for diluting serum 2% PEG was added.

7.4   Substrate buffer

7.4.1   0.05M ammonium acetate/0.05M citrate buffer

pH 5.5 with 0.1% benzoic acid.

To 800 ml of distilled water add,

Citric acid (M.w. 210.14)	10.5 g
Glacial acetic acid (17.5M)	3.0 ml
Benzoic acid	1.0 g

Adjust the pH to 5.5 with concentrated ammonia. Fill up to 1000 ml with water.

7.4.2   Peroxidase substrate

0-phenylene-diamine-di Hcl (OPD)	15 mg
0.05M ammonia acetate/citrate buffer	15 ml
1% Hydrogen peroxide	0.15 ml