RECENT DEVELOPMENTS

IN THE CONTROL
OF ANAPLASMOSIS, BABESIOSIS
AND COWDRIOSIS

PROCEEDINGS OF A WORKSHOP HELD AT ILRAD
NAIROBI, KENYA
13–15 MAY 1991

Edited by
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THE INTERNATIONAL LABORATORY FOR RESEARCH ON ANIMAL DISEASES
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The International Laboratory for Research on Animal Diseases (ILRAD) was established in 1973 with a global mandate to develop effective control measures for livestock diseases that seriously limit world food production. ILRAD's research program focuses on animal trypanosomiasis and tick-borne diseases, particularly theileriosis (East Coast fever).

ILRAD is one of 18 centres in a worldwide agricultural research network sponsored by the Consultative Group on International Agricultural Research. In 1992 ILRAD received funding from the African Development Bank, the Rockefeller Foundation, the United Nations Development Programme, the World Bank and the governments of Australia, Belgium, Canada, Denmark, Finland, France, Germany, India, Italy, Japan, the Netherlands, Norway, Sweden, Switzerland, the United Kingdom and the United States of America.

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Foreword

This Workshop is the second of two that the International Laboratory for Research on Animal Diseases (ILRAD) convened to assist in its research planning for the quinquennium 1994–1998. The first Workshop, `Recent Developments in the Research and Control of *Theileria annulata',* was held in September 1990.

ILRAD has a global mandate for the improvement of livestock production through improved disease control. The focus of its research since its inception in 1973 has been on tsetse-fly-transmitted trypanosomiasis and on theileriosis caused by *Theileria parva.* At the time ILRAD was established, a method of immunization against *T. parva* had been developed based on infection of animals with live parasites and simultaneous treatment with tetracylines to control the severity of infection. To develop a new non-living vaccine, it was necessary to obtain a detailed understanding of the important protective immune responses of cattle and the parasite antigens that provoked them. This became a major focus of ILRAD's research. ILRAD has also focused on improving the infection-and-treatment method of immunization by addressing the problems posed by the antigenic diversity of *T. parva* stocks and strains found in the field and by characterizing immunizing stocks for use in different countries. To define the tick-borne disease environments in which infection-and-treatment immunization is being used, it has also been necessary to develop more sensitive and specific assays for the diagnosis of tick-borne diseases. In the longer term, the development of a subunit vaccine for *T. parva* will have to be considered in parallel with similar developments for other tick-borne disease pathogens because these diseases are considered together for control purposes. The progress made in research on *T. parva* at ILRAD, particularly in defining the nature of the immune responses, has been of direct relevance to research on other tick-borne diseases. The establishment of a vaccine development area within the Theileriosis Program, investigating the induction of protection using different vectoring and adjuvant systems in cattle, provides another important opportunity for future work on other tick-borne diseases in collaboration with laboratories elsewhere.

It is against this background that ILRAD wished to discuss the importance of anaplasmosis, babesiosis and cowdriosis, the methods of control being used for the diseases and the problems encountered with these control methods, recent findings in the epidemiology and molecular biology of these diseases and developments towards new vaccines. These discussions will help ILRAD identify where its current research program may contribute to research on these diseases and, should funding and mandate allow, the establishment of collaborations that will contribute towards the improvement in control of other tick-borne diseases using non-living, easily delivered vaccines.

In response to a recommendation from this Workshop on collaboration in the development of standardized diagnostic tests, representatives from the Centre for Tropical Veterinary Medicine (Edinburgh), ILRAD, the Tick Fever Research Centre (Brisbane), University of Florida and Washington State University met to discuss the various tests being developed for anaplasmosis and babesiosis. A collaboration and sharing of reagents was arranged to identify the most effective reagents for incorpo-

ration in both antigen- and antibody-capture enzyme-linked immunosorbent assays (ELISAs). A promising test described at the Workshop was a competitive ELISA for *Cowdria ruminantium* based upon a 32-kilodalton (kDa) antigen. That promise has since been undermined by widespread cross-reactivity with other rickettsia. It is hoped that the widely recognized need for diagnostic tests and the collaboration described above will result in the rapid development of such tests.

Since the Workshop, ILRAD's Board of Directors and the Technical Advisory Committee of the Consultative Group on International Agricultural Research (CGIAR), which sponsors ILRAD, have approved modification of the institute's research objectives to include the development of novel vaccines for other tick-borne diseases. This change in objective will be undertaken on a collaborative basis with laboratories already working on identifying potentially protective antigens. ILRAD's vaccine development and bovine immunology expertise and laboratory resources will be its contribution to these collaborations.

I am particularly grateful to Mr. Peter Werehire for his assistance in preparing these proceedings. I would like to thank Mr. Kepher Nguli for helping to organize and run the workshop. I also thank my ILRAD colleagues for acting as rapporteurs and chairmen and for reading and commenting on manuscripts. Ms. Susan Nduta retyped many of the manuscripts and the rapporteurs' summaries. Mr. William Umbima and the ILRAD library staff checked the references. The maps of South America and West Africa were prepared by Mr. Russ Kruska and Mr. Mohammed Baya, of ILRAD's Socioeconomics Program. The cover illustrations were prepared by Mr. David Elsworth and Figure 2 in Prof. A. Ilemobade's paper was re-drawn by Mr. Joel Mwaura, both of ILRAD's Graphic Arts Unit. The photomicrograph of *Cowdria ruminantium* reproduced on the cover is from a colour slide provided by Dr. Linda Logan-Henfrey. I thank CRC Press Inc., Baton Rouge, USA, for permission to reproduce Figure 2 in the paper by Dr. Sonia Montenegro-James.

Thomas T. Dolan
International Laboratory for
Research on Animal Diseases
Nairobi, November 1992

ORGANIZING COMMITTEE
of the ILRAD Workshop on Recent Developments
in the control of Anaplasmosis, Babesiosis and Cowdriosis
13–15 May 1991, Nairobi, Kenya:

T.T. Dolan, J.J. Doyle, S.P. Morzaria,
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Opening address

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It is my pleasant duty to welcome you, put this workshop on `Recent Developments in the Control of Anaplasmosis, Babesiosis and Cowdriosis' into context, stress what ILRAD needs to get from it and then hand over to the chairman of the first session to get the meeting moving.

I am very pleased to welcome you all this morning. Almost every one of the invited participants has made it to the opening session, in many cases after lengthy journeys. I think a majority of you have been to ILRAD before and `welcome back' seems appropriate. I am also very pleased to see some first-time visitors and we will be doing our best to help you find your way around and to ensure you enjoy your visit to Kenya.

ILRAD is supported by the Consultative Group on International Agricultural Research to develop improved methods for the control of livestock diseases that adversely affect agricultural productivity in developing countries. Most of you know that the Laboratory has concentrated to date on the African tsetse-transmitted trypanosomiasis problem and on East Coast fever, or theileriosis transmitted by ticks and caused by *Theileria parva.* Most of you also know that ILRAD works on a five-year planning, budgeting and review cycle, and that is what brings us together here this week.

Left to ourselves, we are probably quite capable of drawing up plans for the period 1994–1998 for the continuation of our current research programs on trypanosomiasis and theileriosis, and possibly getting them funded. But we are well aware that it will be necessary to improve both our understanding and the control of a number of additional tick-borne diseases, including anaplasmosis, babesiosis and cowdriosis, to get the maximum benefit from any future improvement in the control of East Coast fever. We need to know what progress other laboratories are making towards the control of these three diseases. This is not to say that we are planning to start work on anaplasmosis, babesiosis or cowdriosis but we need to review what is going on in these areas when we make the case for continuation of work on theileriosis. This we explained when we wrote earlier inviting your participation this week, and this is where we need your help.

The group here this morning includes leading figures in research and control of anaplasmosis, babesiosis and cowdriosis and some specialists who have worked on field aspects of these diseases in Africa. If you are not familiar with research progress in these areas, I do not know who is! At this point I would therefore like to restate the purpose of the workshop, which is:

`To obtain up-to-date information on the global distribution, control methods and economic importance of anaplasmosis, babesiosis and cowdriosis; insights into

current research on the biology, new diagnostics and vaccine development; and to examine what new biological control materials and diagnostics might become available commercially in the near future.'

The workshop will provide an opportunity to identify where ILRAD might compliment current research and identify neglected areas that might become primary research foci in the future. The workshop has been organized around a number of themes and rapporteurs have been appointed to summarize the status of knowledge and anticipated discussion in each area. The rapporteurs will summarize the gist of presentations, and note significant points arising in discussion and particularly any gaps in knowledge or recommendation for further work.

I hope the session chairman will help the group to stay focussed on the various objectives and I look forward to having your help in this important planning task. I am pleased to declare the workshop opened and to hand over to the Chairman, Bob Dalgliesh, to get the first session which is to review the status of these three diseases under way.

DISEASE DISTRIBUTION, ECONOMIC IMPORTANCE AND CONTROL

Distribution, economic importance and control measures for *Babesia* and *Anaplasma*

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INTRODUCTION

Bovine babesiosis and anaplasmosis form part of a complex of diseases sharing the feature of being predominantly transmitted by ticks. Worldwide, babesiosis and anaplasmosis must count amongst the most important of all tick-borne diseases (TBDs). They are impediments to the development of livestock industries (McCosker, 1981). A total of 111 species have been described from the genus *Babesia* and at least seven of these are known to affect cattle (Levine, 1988). Three species, *Babesia bovis, B. bigemina* and *B. divergens,* are recognized as being of economic significance in cattle. In the genus *Anaplasma,* only *Anaplasma marginale* infection is important in cattle. Diseases caused by these four organisms form the basis of this paper but, because of its lesser significance, *B. divergens* will be mentioned briefly.

PARASITES AND DISTRIBUTION

The vectors and distribution of *Babesia* species and *A. marginale* are summarized in Table 1. *Babesia bovis, B. bigemina* and *A. marginale* are present in many countries between 40 ⌈N and 32 ⌈S (McCosker, 1981). *Boophilus microplus* is the most important and widespread vector but its distribution in Africa is limited, even though much of the continent is climatically favourable for its survival. What stops it from spreading is the overlapping distribution of *Boophilus decoloratus,* a closely related tick that favours the drier and colder parts of the potential range of *Boophilus microplus.* The two tick species interbreed but produce sterile progeny which creates a hybrid zone through which *Boophilus microplus* penetrates with difficulty, except in high rainfall areas (Sutherst, 1987).

Ba*besia bigemina* is transmitted by the same tick species as *B. bovis* and therefore has much the same distribution except in Africa, where it is more widespread because of the ability of *Boophilus decoloratus* and *Rhipicephalus evertsi* to act as vectors (Friedhoff, 1988).

Anaplasma marginale is transmitted biologically by at least 20 tick species. Mechanical transmission by biting arthropods also occurs and is considered to be

TABLE 1. The vectors and distribution of *Babesia* species and *Anaplasma marginale* of cattle.

Species	Vector	Distribution
Babesia bovis	*Boophilus microplus*	Latin America
	Boophilus annulatus	Southern Europe
	Boophilus geigyi	Africa
		Asia
		Australia
Babesia bigemina	*Boophilus microplus*	As for *Babesia*
	Boophilus decoloratus	*bovis*, more wide-
	Boophilus annulatus	spread in Africa
	Boophilus giegyi	
	Rhipicephalus evertsi	
Babesia divergens	*Ixodes ricinus*	Northern Europe
	Ixodes persulcatus	
Anaplasma marginale	*Boophilus* spp.	As for *Babesia*
	Rhipicephalus spp.	*bigemina*, more
	Hyalomma spp.	widespread in
	Dermacentor spp.	Africa and South
	Tabanidae	America, also in
	Stomoxys spp.	North America
	Psorophora spp.	

the major method of transmission in the southeastern USA (Kuttler, 1979). In Argentina, the occurrence of *A. marginale* well beyond the limit of tick distribution is also ascribed to mechanical transmission (Spath *et al.,* 1990). However, in Australia mechanical transmission of *A. marginale* apparently plays no major role in the epidemiology of anaplasmosis (Callow, 1984).

Babesia divergens is transmitted almost exclusively by *Ixodes ricinus* in northern Europe (Friedhoff, 1988). It has undoubtedly been exported to other parts of the world, including Africa, Australia and North America where different *Ixodes* species occur but, so far, there has been no evidence of it becoming established in these other environments.

ECONOMIC IMPORTANCE

Up to 500 million cattle are exposed to babesiosis and anaplasmosis the world over but this figure is not a true reflection of the number at risk to disease. The majority of native *Bos indicus* and Sanga type cattle in endemic regions are probably exposed to *B. bovis, B. bigemina* and *A. marginale* infections but do not develop overt disease. This is partly due to the existence of a state of enzootic stability whereby the cattle become naturally infected at an early age, when there is significant

passively acquired and innate immunity, and are immune to challenge later in life. Although the infections can have a serious effect on previously unexposed adult cattle, these breeds are generally more resistant than *Bos taurus* breeds (Callow, 1984), presumably because of a long association between host and parasite.

Exposure of improved *Bos taurus* cattle can, however, have disastrous consequences, especially under the following conditions:

(a) When susceptible, high-risk cattle such as bulls and pregnant cows are imported into endemic areas, mortality rates in excess of 50% are not uncommon (McCosker, 1981). In a recent shipment of about 1,200 New Zealand cattle to Indonesia, a mortality rate in excess of 70% was recorded (R.C. Payne, unpublished data). It has been estimated that about 70,000 susceptible breeding cattle are being imported into enzootic areas of the world each year (P.D. Chudleigh, unpublished data).

(b) When cattle are exposed following the spread of ticks into previously uninfested areas. An extreme example of this was the loss of one million cattle in Zimbabwe due to tick-borne and other diseases following disruption of dipping during the 1970s (Lawrence *et al.,* 1980).

(c) When infection is introduced into a disease-free vector population. There are few countries or areas where the vectors of *Babesia* and *Anaplasma* are present but not the organisms. One such country, New Caledonia, has been endemic for *Boophilus microplus* for many years but only recently became infected with *Babesia* following the introduction of Australian cattle carrying these parasites.

(d) When enzootic stability fails to develop due to low tick transmission rates caused by, amongst others, ecological factors such as drought or use of acaricides. In Australia, the naturally low transmission rate of *B. bovis* also contributes to most outbreaks occurring in cattle bred in endemic areas (Callow and Dalgliesh, 1980).

The economic impact of babesiosis and anaplasmosis can be expressed in terms of mortality, loss of production (including liveweight gain, milk production and draught potential), the cost of control and, in some cases, restrictions placed on the movement of animals (Norval *et al.,* 1991). As in the case of other areas of pest management, there are few reliable estimates of economic losses resulting from babesiosis and anaplasmosis. The problem is further complicated by the occurrence of more than one tick-borne disease in most areas and the need to control ticks *per se.*

The relative importance of *B. bovis, B. bigemina* and *A. marginale* differs in different parts of the world. In North and South America and southern Africa, *A. marginale* is the most important by virtue of its wider distribution and high virulence. In Australia it has the same distribution as *Babesia* and is only moderately virulent, accounting for no more than 10% of recorded outbreaks of tick-borne diseases. When present, *B. bovis* is usually an important cause of disease. In Australia, for instance, it accounts for 80% of all outbreaks of tick-borne diseases. *Babesia bigemina* is generally less virulent and therefore less important except in areas where it is the only *Babesia* spp. present. In Australia, it is the cause of only about 10% of confirmed babesiosis outbreaks.

CONTROL

In most countries where babesiosis and anaplasmosis are endemic, disease control rather than eradication is the only realistic option. Eradication is unlikely to be feasible except in ecologically isolated areas and in advanced countries with the necessary resources. This is particularly true in the case of anaplasmosis with its non-bovine reservoirs and variety of vector species.

Active control of babesiosis and anaplasmosis is achieved by three main methods: immunization, chemoprophylaxis and vector control. Ideally, the three methods should be integrated to make the most cost-effective use of each and also to exploit breed resistance and the development and maintenance of enzootic stability (Lawrence and de Vos, 1990).

Live *Babesia bovis* and *Babesia bigemina* vaccines

Live vaccines prepared from blood of infected cattle have been used since the beginning of the century to protect cattle against babesiosis. In the early carrier-donor systems, blood from recovered cattle served as vaccine. Today, improved production techniques provide more refined and effective vaccines. Commercially available vaccines in Australia and elsewhere are still based on the blood of infected, splenectomized calves (Callow, 1984), but current work will determine the production potential of *in vitro* culture systems for both *B. bovis* and *B. bigemina* (Jorgensen *et al.,* 1992). Available vaccines are generally effective and safe, provided care is taken to ensure viability at the time of use and use is restricted to low-risk classes of cattle (young animals). These precautions are essentially the same as those for other live vaccines.

As with any vaccine production facility, a satisfactory code of manufacturing practice applies to the production of babesiosis vaccines. This code must ensure adequate quality control, absence of contaminants and delivery of safe, efficacious products. The maintenance of these standards requires facilities, funding and infrastructure unavailable in most countries in need of effective vaccines. Therefore, controlled production systems are bound to develop which satisfy the needs of entire regions rather than individual countries. It was with this in mind that FAO identified a government laboratory in Malawi for support in developing a production facility to service the needs of southern African countries. Production statistics from existing laboratories generally reflect demand rather than production potential. Thus, in Australia, where the demand for *B. bovis* vaccine has been relatively constant at 0.7–0.8 million doses each year, the current production potential with existing facilities is at least twice this figure. Chilled vaccine is popular in some countries, including Australia, but has not been widely accepted elsewhere because of shortcomings, including short shelf-life, difficult quality control and risk of vaccine-induced reactions in adult cattle (Dalgliesh, 1992).

The risk of vaccine-induced reactions in adult cattle has been reduced with the development and use of attenuated strains. Attenuation of *B. bovis* by serial passage in splenectomized calves is well documented (Callow, 1984) and may result from selective enrichment of avirulent parasite sub-populations (Carson *et al.,* 1990).

Babesia bigemina parasites with low, stable virulence have been obtained by exploiting observations of early workers that parasites from chronic infections are less virulent than acute phase parasites. Thus, relapse parasites collected following splenectomy of carrier animals are now used to prepare a *B. bigemina* vaccine (Callow, 1984; Pipano and Hadani, 1984). Long-term maintenance in culture containing horse serum and the use of sublethal doses of irradiation have also been used in attempts to reduce the virulence of *B. bovis* and *B. bigemina* (Dalgliesh, 1992). Even with relatively avirulent strains, there is a risk of reactions when adult cattle of susceptible breeds have to be immunized. Attempts to reduce this risk include the prophylactic use of drugs in what is sometimes referred to as chemoimmunization. The most notable success in this field has been the use of oxytetracycline given at or soon after vaccination to modify *Babesia* reactions (Pipano *et al.,* 1987; W.K. Jorgensen, unpublished data). Some success has also been achieved by immunizing cattle with non-living, culture-derived antigens before inoculation with a live *B. bovis* vaccine (Pipano *et al.,* 1984).

Antigenic variation occurs within and between strains of *B. bovis* and *B. bigemina* (Dalgliesh, 1992) but it does not appear to affect the level or duration of protection afforded by live vaccines. A single inoculation usually confers lasting immunity against heterologous challenge, even in different countries. Thus, Australian strains were protective against experimental challenge in South America, South Africa and Sri Lanka (Dalgliesh *et al.,* 1990) and have successfully protected thousands of susceptible cattle imported into Southeast Asian and South American countries.

Even though living vaccines against babesiosis are generally highly effective, failures to protect cattle do occur. This happened in Australia in 1968 and 1976 due to loss of immunogenicity of particular *B. bovis* vaccine strains passaged repeatedly in splenectomized calves (Callow and Dalgliesh, 1980). Since 1977, passaging of the vaccine strain has been restricted by regular reversion to stabilate with a low passage number. This approach kept the failure rate at a low level for ten years. Since 1988, however, the number of failures has again increased, especially in well-managed *Bos taurus* cattle (Bock *et al.,* 1991). There was no correlation between vaccine failures and the time after immunization. Controlled testing of the vaccine strain against a virulent heterologous isolate at regular intervals since 1980 indicated its protective effect had not changed. However, the vaccine strain was poorly protective against three recent isolates from forms which had reported vaccine failures (Bock *et al.,* 1991). These results and field observations indicating regional spread of parasites causing failures suggest that the latest failures stem from changes in field populations, not the vaccine strain.

Genetic exchange and recombination take place in *Plasmodium falciparum* and may also occur in *Babesia* (Walliker, 1989). Both species therefore appear to be populations of interbreeding organisms in which new genotypes are continually generated. This possibility led Walliker (1989) to predict that widespread use of a vaccine based on a single form of a given antigen may result in rapid selection of parasites expressing alternative antigen forms. Recent experience in Australia seems to indicate that this selection will take place even when antigenically complex live vaccines are used. If this theory is correct it may mean that the useful life of a live vaccine strain of *B. bovis* will depend on the degree of exposure of field populations to immunized animals, and will be ten years or less if exposure is frequent.

Live *Babesia divergens* vaccines

A standardized *B. divergens* vaccine is prepared in Sweden from the blood of infected, splenectomized calves in much the same way as the *B. bovis* vaccine in Australia. In Germany, blood of infected gerbils *(Meriones unguicalatus)* has also been used in small-scale immunization trials and has the advantage of being free of bovine cells (Dalgliesh, 1992). Strains used in the Swedish and German vaccines are not attenuated but the trend has been to immunize calves before they are 12 months old, when natural resistance is evident (Christensson and Thorburn, 1987). As reviewed by Dalgliesh (1992), efforts to attenuate *B. divergens* by rapid passage in splenectomized calves, irradiation and passage in abnormal hosts (gerbils) have all been unsuccessful but a reduction in virulence was seen following 18 months in culture.

Live *Anaplasma* vaccines

Anaplasma centrale is a relatively benign organism which confers partial protection against challenge with virulent *A. marginale.* It was isolated in South Africa in 1911 (Potgieter, 1979) and has been used for years to immunize cattle in many countries. The level of protection afforded by *A. centrale* is generally adequate against moderate challenge in Australia (Callow and Dalgliesh, 1989) but deficient against virulent challenge in Latin America and elsewhere (Palmer, 1989). A vaccine based on an *A. marginale* strain attenuated by irradiation and passaged through deer and sheep (Palmer, 1989) has also been used successfully to immunize cattle. This vaccine is commercially available in Latin America and California (Corrier *et al.,* 1985) and affords solid protection against virulent challenge.

Significant differences exist between some isolates of *A. marginale* as shown by differential reactivity with isolate-restricted monoclonal antibodies and limited cross-protective immunity (McGuire *et al.,* 1984; Palmer, 1989). The ability of *A. marginale* to generate antigenic variants appears to have been confirmed recently with the demonstration of cyclical multiplication of organisms during persistent infection of immunocompetent hosts (Kieser *et al.,* 1990). Interestingly, there has been no field evidence of changing levels of protection following immunization with *A. centrale,* even though its use as a vaccine has been commonplace in South Africa since 1911 and in Australia since 1974.

Non-living vaccines

Non-living vaccines would overcome difficulties inherent in the production, transportation and use of live vaccines. Most attempts to develop non-living *Babesia* and *Anaplasma* vaccines ended while still in the experimental stage but there have been some notable successes. Probably the best example is the commercially available *Anaplasma* vaccine based on a lyophilized preparation of organisms administered with an adjuvant. Yearly boosters of this vaccine are recommended and the level of protection afforded is dependent upon the nature of the challenge isolate (Palmer, 1989). The potential for this vaccine to induce isoerythrolysis in the new-born is

well-known and efforts have been made to minimize the risk by, amongst others, improved methods of antigen purification (Palmer, 1989).

Soluble *B. bovis* antigen prepared from culture supernatant and given with an adjuvant has also been used to immunize cattle. As reviewed by Montenegro-James *et al.* (1989), immunized cattle undergo milder reactions than susceptible controls when challenged with heterologous strains and protection is noticeable for up to 13 months. As a result, production and distribution of the vaccine in Venezuela is due to commence for immunization of susceptible, imported stock. In parallel studies in Australia, the level of protection afforded by culture-derived *B. bovis* antigen was not considered adequate to make a viable proposition.

Subunit vaccines

Progress made in recent years towards defined subunit vaccines against *Babesia* and *Anaplasma* is described elsewhere in these proceedings. However, the evidence provided earlier for the evolution of antigenically different *B. bovis* field isolates in the face of regular use of a live vaccine in Australia is worthy of note. The vaccine strain used comprised several sub-populations with variable biological and genomic characteristics (Carson *et al.,* 1990; Timms *et al.,* 1990).

Chemotherapy/chemoprophylaxis

Several groups of compounds have been used in the chemical control of babesiosis (Kuttler, 1988). Of these, only imidocarb dipropionate, diminazene aceturate and tetracycline antibiotics remain freely available in most endemic countries. Both imidocarb and oxytetracycline are effective for the control of anaplasmosis as well. Chemoprophylaxis is not a viable long-term alternative to effective immunization, but imidocarb and diminazene have been used to protect cattle for several months against babesiosis and low dose administration of tetracycline in the feed has been suggested as a means of controlling anaplasmosis in the USA.

The long prophylactic effect of imidocarb against *B. bovis* and *B. bigemina* has been used to protect newly introduced cattle in the hope that the animals will become exposed to natural infections and develop immunity while still partially protected by the drug. This procedure has been used with some success in instances where vaccines are not readily available but has major limitations. The transmission rate by ticks must be very high to ensure infection during the period of protection and the sheer numbers of ticks required are likely to affect the cattle. Furthermore, immunity conferred in this way may not always be adequate to protect cattle against future challenge (de Vos *et al.,* 1986).

Vector control

Vector control was used successfully to control and eventually eradicate *Babesia* from the USA. In Africa, where babesiosis and anaplasmosis form part of very important

complexes of ticks and tick-borne diseases, the principle means of control has also been based on intensive, usually government regulated, tick-control programs. However, evidence is mounting which indicates that tick control as a sole means of disease prevention is not a viable long-term option, mainly due to the development of acaricide resistance and economic constraints.

Integrated control

No single method is likely adequately to control the complex problem of these and other tick-borne diseases in Africa and elsewhere (Norval *et al.,* 1991; Young *et al.,* 1988). However, there are now clear grounds for considering new, integrated approaches which encompass:
    (a) selection of tick-resistant cattle,
    (b) exploitation of enzootic stability,
    (c) use of effective vaccines when enzootic stability is not evident, and
    (d) use of acaricides only when economically justified in relation to the direct effects of ticks on livestock production.

CONCLUSIONS

Most of the several approaches used to control disease caused by *Babesia* and *Anaplasma* have involved induction and maintenance of immunity to challenge. Control strategies planned for the foreseeable future must continue to include some form of immunization, but none of the currently available vaccines is ideal. The effort invested in the research and development of better alternatives is considerable. However, lessons learnt in Australia with live vaccines and the experience of our colleagues working on malaria show that we need to improve our understanding in two important areas if we are ever to construct the ideal *Babesia* vaccine. The same may also be true for an *Anaplasma* vaccine. Firstly, we must better understand factors which cause variant characteristics to arise in parasite populations, and develop ways for predicting and measuring these changes. The possible role of genetic exchange among field populations and parasites used in vaccines should be considered. Secondly, the expression of protective immunity to *Babesia* and *Anaplasma* infections by the host is still poorly understood and we lack means of measuring it. If malaria research can be used as a guide, the search for improved *Babesia* and *Anaplasma* vaccines is bound to fail if it is based solely on sound (molecular) biological principles without understanding the mechanisms of immune-expression against the organisms.

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Distribution, economic importance and control measures for *Cowdria ruminantium*

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INTRODUCTION

Heartwater is a rickettsial disease of ruminants caused by *Cowdria ruminantium* and transmitted by ticks of the genus *Amblyomma.* The most important vectors of heartwater to livestock are the tropical bont tick *Amblyomma variegatum* and the southern Africa bont tick *A. hebraeum.* Other vectors that frequently parasitize livestock are *A. asterion, A. cohaerens, A. gemma, A. lepidum* and *A. pomposum.* Heartwater occurs in sub-Saharan Africa and has been introduced with *A. variegatum* to several islands in the Indian and Atlantic oceans and to a number of islands in the Caribbean. The presence of heartwater and *A. variegatum* in the Caribbean constitutes a threat to livestock production in the tropical and sub-tropical areas of the American mainland (Barré *et al.,* 1987). Heartwater is considered to be the most important tick-borne disease of cattle in southern Africa (Howell *et al.,* 1981) and in eastern Africa it is ranked second in importance to East Coast fever; its economic importance in cattle in the remainder of sub-Saharan Africa is recognized but not well documented. Unlike the other major tick-borne diseases of cattle (anaplasmosis, babesiosis and theileriosis), heartwater is also of considerable economic importance in sheep and goats and it can cause mortality in several wild ruminant species (Oberem and Bezuidenhout, 1987a). Heartwater has traditionally been controlled by the dipping of cattle and sometimes sheep and goats (i.e., vector control), and an infection-and-treatment method of immunization has been available in South Africa since the 1940s. Endemic stability for heartwater probably occurs in most situations in which indigenous livestock are maintained without tick control in infected areas.

From the turn of the century until the 1960s almost all the research on heartwater was carried out in South Africa, mainly at the Veterinary Research Institute, Onderstepoort. This situation started to change as the importance of the disease became more widely recognized in Africa, and when the spread of *A. variegatum* in the Caribbean was noted. The research effort gained momentum in the 1980s, with the development of an *in vitro* culture system for *C. ruminantium* (Bezuidenhout *et al.,* 1985) and the application of new research methods based on molecular biology. Research on heartwater and its vectors is now carried out by several groups in Africa and elsewhere. These include:

- The University of Florida, which has a Southern Africa Development Coordination Conference (SADCC) regional project based in Zimbabwe.
- Washington State University, which has a project in Kenya.
- The Veterinary Research Institute at Onderstepoort in South Africa.
- Institut d'Elevage et de Médecine Vétérinaire des Pays Tropicaux of France, which conducts research on the Caribbean island of Guadeloupe.
- The Heartwater Research Network, Faculty of Veterinary Medicine, University of Utrecht in The Netherlands.
- The Food and Agriculture Organization of the United Nations at the regional project, `Production of *Anaplasma, Babesia* and heartwater vaccines for East and Central Africa', in Lilongwe, Malawi.
- Universidade Eduardo Mondlane in Maputo, Mozambique.

The single most important development in heartwater research in the past decade has undoubtedly been the *in vitro* culture of *C. ruminantium,* which has now been refined by Yunker *et al.* (1988) and Byrom and Yunker (1990) so that large quantities of the organism can be produced routinely. This source of *C. ruminantium* has facilitated the development of the first reliable diagnostic tests that can be used to detect the organism or antibodies to it in living hosts and the organism in the tick vector. These tests are now being used in field studies on the epidemiology and economics of heartwater, which should provide a basis for improving the control or management of the disease in the future. The culture system also provides a source of *C. ruminantium* for immunological studies, which may lead to the production of improved vaccines. Such vaccines could be based on culture-derived organisms or on specific antigens produced by recombinant-DNA technology. Jongejan (1991) has already reported protective immunity to heartwater after vaccination with an *in vitro*-attenuated stock of *C. ruminantium.* Further research on vaccine development is being undertaken by the University of Florida/USAID/SADCC Heartwater Research Project in Zimbabwe.

Recent research on the biology and ecology of *Amblyomma* ticks is also contributing to the understanding of the epidemiology of heartwater and has led to a new innovative control method for *A. hebraeum* and *A. variegatum.* Attached males of these species emit an aggregation-attachment pheromone (AAP), which is used by unfed nymphs and adults in host location and host selection (Norval *et al.,* 1989a, 1989b). The effect of the pheromone is to focus heartwater infection in certain host groups such as cattle and buffalo. The ticks that feed on these hosts, which remain long-term carriers (Andrew and Norval, 1989), become highly infected (Norval *et al.,* 1990) and play an important role in the maintenance of endemic stability. The chemical components of the AAP have been identified (Schoni *et al.,* 1984; Apps *et al.,* 1988; Lusby *et al.,* 1991).

DISTRIBUTION

The distribution of heartwater corresponds closely with that of its African *Amblyomma* vectors (Provost and Bezuidenhout, 1987). Distribution maps of the vectors have been

compiled by Walker and Olwage (1987). The most widely distributed vector in Africa is *A. variegatum,* which occurs throughout western Africa from the Sahel southwards, in areas of northeastern Africa including southern Sudan, most of Ethiopia and northwestern Somalia, in most of central and eastern Africa, and in southern Africa as far south as the Zambezi River system and central Mozambique. In the highland areas of northern Angola, *A. variegatum* gives way to *A. pomposum* and in southern Africa it is replaced by *A. hebraeum.* There is evidence that *A. variegatum* and *A. hebraeum* are mutually exclusive due to inter-specific competition resulting from inter-specific responses to the female-produced sex pheromone and the male-produced AAP (Rechav *et al.,* 1982; Norval, 1983a). *Amblyomma cohaerens, A. gemma* and *A. lepidum* occur fairly widely in eastern and northeastern Africa. *Amblyomma asterion* occurs in the Congo basin, in coastal areas of Angola and on the off-shore islands of Sao Tome and Principe.

Outside the African continent, *A. variegatum* is established in Yemen, on the Indian Ocean islands of Madagascar, Grande Comore, Reunion and Mauritius, on the Atlantic Ocean islands of Cape Verde, and on the Caribbean islands of Guadeloupe, Marie Galante, Martinique, Antigua, St. Lucia, St. Kitts, Nevis, St. Maarten/St. Martin, La Desirade, Monserrat and Dominica. It has also been recorded from or has been temporarily established on several other islands in the Antillean chain—Anguilla, Barbados, Puerto Rico, Saba, St. Eustatius, St. Vincent, the US Virgin Islands and Vieques.

Three other African *Amblyomma* species, *A. marmoreum, A. sparsum* and *A. tholloni,* are known to transmit heartwater, but are not considered to be important vectors to livestock as they are parasites of specific wild hosts. *Amblyomma marmoreum,* a tortoise tick, may be an exception in parts of South Africa where its immature stages frequently parasitize domestic animals (Petney and Horak, 1988).

Two American *Amblyomma* species, *A. maculatum* and *A. cajennense,* have been shown to be vectors of heartwater under experimental conditions (Uilenberg, 1982a, 1983a) but it is not known if the species would be able to perpetuate infection under field conditions. Field vectors must be capable of onward transmission from asymptomatic carriers; to date, the capacity to transmit from carriers has been demonstrated only for nymphs of *A. hebraeum* (Andrew and Norval, 1989) and *A. variegatum* (Jongejan *et al.,* 1988). *Amblyomma maculatum* occurs in coastal areas around the Gulf of Mexico and *A. cajennense* in Central America, Cuba and much of South America as far south as Uruguay. If either or both of these species are found to be potential field vectors, large areas of the American mainland could be at risk to heartwater introduced by the importation of carriers. As yet such an introduction has not occurred despite the importation of wild ruminants from Africa (potential carriers) into some infested areas, particularly in the southern United States.

The most serious threat of heartwater to the American mainland is undoubtedly the presence of *A. variegatum* and *C. ruminantium* in the Caribbean (Barré *et al.,* 1987). The tick is continuing to spread in the Caribbean, infesting about one new island each year (Alderink and McCauley, 1988). Should it be introduced to the mainland, *A. variegatum* has the potential to become established and spread over a large area in the southern United States, Central America and a large part of eastern South America. Sutherst and Maywald (1985) have shown, using the climate-match-

ing model CLIMEX, that these areas are climatically suitable for the survival of *A. variegatum*.

ECONOMIC IMPORTANCE

The distribution of the disease and its tick vectors places upper limits on the number of animals currently at risk to heartwater. Using FAO/WHO/OIE (1990) estimates, Africa has a maximum of about 175 million head of cattle that are either in countries where heartwater has been confirmed (Provost and Bezuidenhout, 1987) and/or where vectors occur and the disease is likely to be present (Walker and Olwage, 1987). This figure includes cattle on the Indian and Atlantic ocean islands where the disease is established. Caribbean islands on which *A. variegatum* is established mostly have small numbers of cattle, ranging from 7,000 head on St. Lucia and Nevis to 74,000 head on Guadeloupe (FAO/WHO/OIE, 1990). Small cattle populations do not mean that the cattle are economically unimportant to either the individual owner or the state economy. The economic impact of the disease on these animals at risk can be separated into two categories: disease-related mortality and non-lethal losses. Losses in each category include:

Mortality

- of susceptible animals moved to endemic areas,
- caused by heartwater epidemics when vectors spread,
- in endemically unstable situations,
- when tick control fails, and
- when susceptible animals are raised in endemic areas.

Non-lethal losses

- production losses caused by sub-clinical (carrier state) infections,
- losses due to the costs of control of heartwater and its vectors and the treatment of clinical cases,
- lost production potential caused by the inability to upgrade local livestock by cross-breeding with exotic breeds imported into a heartwater endemic area, and
- production losses caused by infestations of vectors.

The circumstances in which heartwater causes mortality in domestic livestock are now well defined. The following are examples of such losses.

Cattle, sheep and goats are moved from heartwater-free to endemic areas. This has occurred when high-yielding exotic breeds have been introduced to upgrade or replace local stock (Uilenberg, 1982b). In Botswana this occurs when cattle are moved from heartwater-free areas in the drier western part of the country to endemic areas in the east (Windsor, 1987). In Zimbabwe it occurs when cattle are moved from

the heartwater-free highveld to the lowveld (Norval and Lawrence, 1979). In South Africa losses have occurred when animals have been moved locally to better grazing (Neitz, 1967). Losses have been reported in Mali when livestock have been trekked from the Sahel to markets in areas where *A. variegatum* is common (Logan *et al.,* 1988).

Heartwater vectors and infection spread to new areas. This is well illustrated by the spread of *A. hebraeum* and heartwater into the Eastern Cape Province of South Africa in the nineteenth century, which caused devastating livestock losses to British settler farmers (Lounsbury, 1899). More recently very large losses of cattle, sheep and goats due to heartwater occurred when the distribution of *A. hebraeum* in Zimbabwe expanded when dipping was disrupted during the pre-independence war (Norval, 1979; Lawrence *et al.,* 1980).

Vector populations are suppressed by tick control measures or drought and endemic stability is lost. The adverse effects of intensive dipping and periodic droughts on endemic stability for heartwater have long been recognized in South Africa (Howell *et al.,* 1981; Bezuidenhout and Bigalke, 1987) but quantitative data on losses have not been published. In Zimbabwe, Norval and Lawrence (1979) reported that over an eight-year period one commercial ranch in an endemic area that practised intensive dipping lost 933 out of 116,038, or almost 1%, calves to heartwater.

Intensive tick control fails or is relaxed. Intensive tick control may fail for a variety of reasons, ranging from acaricide resistance in tick populations to political or economic instability or poor management (Lawrence *et al.,* 1980; Norval, 1983b) or it may be deliberately relaxed, for example following ECF immunization (Uilenberg *et al.,* 1978; Uilenberg, 1984; Radley, 1984). In all these circumstances heartwater can be a major cause of mortality in livestock.

Heartwater-susceptible breeds of livestock are raised in endemic areas. Certain livestock breeds, for example Merino sheep and Angora goats, are particularly susceptible to heartwater and suffer significant losses, even when they are raised in endemic areas (Thomas and Mansvelt, 1957; Gruss, 1987). In endemic areas in the Eastern Cape Province of South Africa, heartwater mortality rates in Angora goats may be up to 30% per annum (Norval, 1974). Van de Pypekamp and Prozesky (1987) reviewed literature which claimed that Merino sheep have heartwater mortality rates of 5–95%.

Although the circumstances in which mortality occurs are well defined, the actual numbers of deaths caused by heartwater have never been studied or recorded in a systematic or structured manner. The estimates of mortality are largely anecdotal, such as those listed by Camus and Barré (1988) and others mentioned above. Some deaths due to heartwater are recorded in the annual reports of veterinary departments and others can be found in the detailed records kept by a small proportion of commercial farmers, but most appear to go unreported. Two important factors contribute to this: firstly, the difficulty of obtaining a definitive diagnosis of heartwater which until the present has only been possible by examination of brain smears and, secondly, heartwater is not a notifiable or specified disease in any country and so farmers and veterinarians are under no obligation to report cases. Despite the lack of reliable estimates, the available information provides a clear indication that heartwater-related deaths are a significant cost to livestock owners.

There are no data concerning non-lethal production losses caused by heartwater. Losses due to the sub-clinical (carrier state) forms of the disease could include slower rates of liveweight gain, delayed or missed conceptions, higher rate of abortions, lower birth weights, and reduced milk yields. Other non-lethal losses are the costs of controlling heartwater and its vectors and in treating clinical cases. Reliable estimates of these costs are difficult to obtain. A small survey of large-scale ranchers in Zimbabwe's southeast lowveld region showed that farmers pay from US$1 to 17 per head per year to dip cattle to protect them from heartwater (Meltzer, 1990). Not only are these costs significant in local currency terms, but since the acaricides and drugs used for treatment have to be imported, they can represent a notable expenditure of foreign currency. In Africa, foreign currency is a scarce resource, and the costs of controlling and treating heartwater represent a distinct opportunity cost in terms of alternative uses for that foreign currency. Thus, the `shadow price' or `true cost to society' of importing acaricides and drugs to control heartwater can be considerably greater than just the `shelf price' of these items.

The lost production potential caused by the inability to import live animals to conduct cross-breeding programs to upgrade the production of indigenous breeds is another opportunity cost of unknown magnitude caused by heartwater. This loss is particularly important in terms of milk production. The indigenous Zebu and Sanga breeds generally produce about 400–800 kg of milk per lactation versus the 2,000–5,000 kg produced by exotic taurine purebreds and cross-breds (Oliver, 1971; Webster and Wilson, 1980; Wilson, 1968). The removal of constraints imposed by heartwater and other tick-borne diseases could allow increased milk production, with the subsequent improvement in human nutritional status.

Non-lethal production losses in cattle caused by infestations of *A. hebraeum* and *A. variegatum* are more adequately quantified than those caused by the disease itself. Norval *et al.* (1989c) and Pegram *et al.* (1989) have demonstrated that each adult female that completes feeding causes a loss in liveweight gain of 10 g for *A. hebraeum* and 41–60 g for *A. variegatum.*

The disease-related mortality and non-lethal production losses may be tempered by the fact that susceptibility could be altered by age, sex, breed and nutritional status. It is generally accepted that there is a reverse age-related innate resistance to heartwater, with calves being resistant for up to three to four weeks of age (Uilenberg, 1983b; van de Pypekamp and Prozesky, 1987). The case for a sex- or breed-related difference in susceptibility is less clear. Van der Merwe (1987) claimed that a heartwater infection-and-treatment immunization program showed clear differences in mortality due to sex and breed. Unfortunately, van der Merwe's results were not statistically analysed, and the number of deaths reported were exceedingly small (24 out of 2,743 animals immunized). Uilenberg (1983b) felt that there is a breed-related difference in susceptibility, but that ` . . . inherited resistance has nothing to do with Zebu influence'. Uilenberg (1983b) further maintained that the humpless breeds which are raised in heartwater endemic regions, such as the N'Dama and Dahomey, are `highly resistant'. He provided no data that supported such a claim. No literature could be found describing the interaction between nutritional status and the impact of heartwater. Finally, it is noteworthy that, overall, heartwater appears to be a more serious problem in southern Africa where the vector is *A. hebraeum* than in the rest of sub-Saharan Africa and the infected islands where *A. variegatum* is the main or

only vector. There is evidence that the vector species does influence the epidemiology of heartwater. In Sudan, Karrar (1968) reported that heartwater was less of a problem in areas infested with *A. variegatum* than in those infested with other vectors. Similarly, in Zimbabwe, Norval (1983a) noted an apparent absence of clinical heartwater in the northwest where the vector is *A. variegatum* and a severe problem in the south where the vector is *A. hebraeum.* A positive correlation between species of vector and the impact of the disease could explain why Uilenberg (1983b) observed that the indigenous N'Dama and Dahomey breeds from West Africa, where *A. variegatum* is more predominant, are more resistant than breeds from southern Africa. The reasons why heartwater causes more losses when associated with one vector species than another are not known and require investigation.

CONTROL

Vector control

Controlling tick-borne diseases through the use of acaricides to control their tick vectors has been practised in eastern and southern Africa since the turn of the century. While this control method has prevented *Amblyomma* ticks and heartwater from becoming established in the highveld of Zimbabwe (Norval and Lawrence, 1979; R.A.I. Norval, B.D. Perry, M.I. Meltzer, R. Kruska and T.H. Booth, in preparation) and achieved localized heartwater eradication in parts of South Africa (Stampa, 1969) and Kenya (A.S. Young, personal communication), it is fraught with difficulties and does not provide a long-term solution to heartwater problems. Listed below are the major problems associated with the control of heartwater by vector control.

- The costs of acaricides (particularly the foreign exchange component) and their application are becoming too high to be met in several affected countries.
- National tick control programs suffer organizational problems and are vulnerable to political or economic instability.
- Acquired resistance to acaricides in one or more tick species can adversely affect control programs.
- Intensive tick control can create endemic instability for heartwater.
- Control of heartwater by intensive tick control may adversely affect endemic stability for other tick-borne diseases such as babesiosis (this is potentially a problem associated with *Amblyomma* eradication in the Caribbean).
- Tick control has little effect on *Amblyomma* abundance where alternate hosts for the adult stage are present; this is because the aggregation-attachment pheromone (AAP) emitted by attached males causes unfed nymphs and adults to attach in preference to the untreated hosts.
- Dipping or spraying using acaricides pollutes the environment and may result in residues in meat and milk.

A significant advance in the control of *A. hebraeum* and *A. variegatum* has been the utilization of the AAP, naturally emitted by the attached males, to attract unfed nymphs and adults to a source of pesticide (Norval *et al.,* 1989a, 1989b, 1991).

Synthetic pheromone components and an acaricide can be incorporated into slow-releasing plastic `tick decoys', which can be attached to the hosts (Sonenshine and Hamilton, 1989). This slow-release tick decoy system has the following advantages over traditional dipping or spraying.

- It uses less acaricide per head treated and should therefore be less costly and less of an environmental hazard.
- It is long-lasting and removes the need for frequent mustering of animals for dipping or spraying.
- Control is directed specifically at *Amblyomma* ticks and so should not affect the epidemiology of tick-borne diseases other than heartwater.
- The use of the AAP to attract unfed ticks makes effective control of *A. hebraeum* and *A. variegatum* feasible, even in the presence of alternate hosts.

Chemotherapy

The first chemotherapeutic agent found to be effective against heartwater was the sulfonamide drug `Uleron' (Neitz, 1939). Subsequently other drugs, in particular tetracyclines, have been found to be effective (Weiss *et al.,* 1952; van Amstel and Oberem, 1987). Two major problems appear to be preventing the widespread adoption of chemotherapy as a control strategy. First, there often are difficulties in the early diagnosis of the disease when chemotherapy is most likely to be effective (van Amstel and Oberem, 1987). The second problem is the relatively high cost of drugs such as the tetracyclines, in both local and foreign currency terms. To demonstrate the magnitude of potential costs, it has been estimated that administering long-acting oxytetracycline as part of the infection-and-treatment immunization method for East Coast fever will cost US$1.40 per animal (A.W. Mukhebi, personal communication). Other cost estimates, reviewed by Mukhebi *et al.* (1990), range from US$2.50–4.00 per head. Uilenberg (1983b) noted that even using long-acting oxytetracycline to treat heartwater may require two or more treatments, which would substantially increase the cost estimates given above.

Chemoprophylaxis

Purnell *et al.* (1989) reported that susceptible cattle introduced to an endemic heartwater area and given three injections of long-acting oxytetracycline on days 7, 14 and 21 after introduction did not develop clinical disease. Again, a major constraint to the widespread adoption of this methodology is the cost. A variation of administering injections at set intervals is to use slow-release drug implants, such as the doxycycline implant. The latter has been reported to `look promising' when used in Angora goats raised in heartwater endemic areas (B. Fivaz, personal communication). A cost-benefit analysis of such implant technology has yet to be published.

Infection-and-treatment method of immunization

The first practical and effective method of immunization was introduced by Neitz and Alexander (1941) who exploited the innate age-related resistance of calves to immunize them with virulent *C. ruminantium.* The infection-and-treatment method of immunization was developed when Uleron became available as a chemotherapeutic agent (Neitz, 1940). The method has since been refined and tetracyclines have replaced Uleron (van der Merwe, 1987). Infections are produced by intravenous inoculation of whole blood from reacting animals (sheep) or stabilate made from ticks fed on reacting animals (Bezuidenhout, 1981). After inoculation the temperatures of individual animals must be monitored so that treatment can be initiated at or soon after the start of the febrile reaction. Block treatment of herds or flocks is not generally recommended as the incubation period is extremely variable (du Plessis and Malan, 1987). Treatment prior to the febrile reaction may prevent the development of immunity and treatment too late in the reaction can result in death. For best results this method of immunization obviously needs to be carried out under strict veterinary supervision.

The need for veterinary supervision obviously increases the cost of application, which limits the number of farmers successfully adopting the technique. The infection-and-treatment method of immunization is used on a large scale only in South Africa, where approximately 175,000 doses of heartwater `vaccine' are sold annually (Oberem and Bezuidenhout, 1987b). Some other disadvantages of the method are:

- Infected blood or stabilate must be stored on dry ice.
- Infection must be administered intravenously.
- The need to monitor febrile reactions in individual animals limits the number that can be immunized at any one time.
- Deaths frequently occur if procedures are not adhered to strictly.
- Use of whole blood introduces a risk of transmitting other pathogens.
- The procedure, which includes the use of tetracyclines, is costly.
- Some problems exist with cross-immunity between immunizing and field stocks of *C. ruminantium* (du Plessis *et al.,* 1989).

Attenuated cell culture vaccines

The development of an attenuated vaccine for heartwater, to replace the infection-and-treatment method of immunization, would represent a significant advance in practical terms. The recent report by Jongejan (1991) on the protection induced by an *in vitro* attenuated stock of *C. ruminantium* is therefore very encouraging. Further research on immunization by this attenuated stock is obviously required; the attenuation of other stocks should also be investigated.

Recombinant vaccines

The ultimate goal of the University of Florida's Heartwater Research Project is to develop a live recombinant vaccinia virus vaccine. The strategy and progress will be discussed in the next section. The reason for the emphasis on recombinant vaccinia virus vaccines is one of economics. The World Health Organization-sponsored smallpox eradication campaign in the 1970s clearly demonstrated that vaccinia virus vaccines can be produced with some very basic tools and materials, freeze dried and distributed to the most remote regions (Fenner *et al.,* 1988). Using production costs from the WHO campaign, it has been estimated that a recombinant vaccinia virus vaccine against heartwater could be produced for as little as US 10 cents per dose (Meltzer, 1990). The ability to freeze-dry vaccinia virus means that there will be no logistical problems associated with establishing a continuous cold or frozen chain from the site of production to the on-farm site of inoculation. Thus, it is feasible to consider that a vaccine against heartwater could be produced and distributed in a developing country without excessive start-up costs.

DISCUSSION

Overall two approaches to the control of heartwater and its vectors are emerging: eradication in the Caribbean and management in Africa.

Barré and Garris (1990) are of the opinion that *A. variegatum* can be eradicated from individual islands in the Caribbean if all livestock and dogs are treated systematically with a suitable acaricide over a given period of time. The two major problems foreseen are ensuring that the acaricide is applied on a regular basis to all potential hosts and the costs of such applications. Slow-releasing tick decoys that are attached to hosts present a possible solution to these problems. The tick pheromone emitted by the decoys will ensure that the unfed ticks are attracted to acaricide-treated hosts; this would not otherwise occur because of the absence of pheromone-emitting male ticks from these animals.

The extent of *Amblyomma* distribution in Africa and the frequent association of *Amblyomma* ticks with wild hosts means that the eradication of heartwater from this continent is simply not feasible. Localized eradication may be possible under some circumstances but, as discussed earlier, it carries considerable long-term risks. The alternative to eradication is the management of heartwater in the environments in which it occurs. In the past the management of heartwater in Africa has been either largely empirical or unplanned.

The first step towards improving the management of heartwater is to collect information on the epidemiology and economic impact of the disease. Currently, by using information on the sizes of tick infestations, the value of the resultant losses in productivity and the costs of tick control, it is possible to estimate a damage threshold for either tick species, above which tick control is economically justified. With the development of an IFA test (Semu *et al.,* 1992), an indirect ELISA (Jongejan *et al.,* 1991) and a DNA probe (Waghela *et al.,* 1991), it should now be possible to conduct surveys to determine the prevalence of heartwater and to carry out controlled experiments to measure the effects of the carrier state on

productivity. With this type of information, and more data on *Cowdria* infection rates in vector populations, it should also be possible to estimate disease transmission thresholds, necessary for the maintenance of endemic stability. Disease transmission thresholds and damage thresholds will form the basis for epidemiological and economic models, with which the effects of a variety of potential control strategies can be simulated. The most appropriate of these strategies for given environments can then be field tested and, if proved effective, can be implemented on a large scale. As improved heartwater vaccines based on either attenuated stocks or specific immunizing antigens produced by recombinant-DNA technology become available, they can be integrated into plans for the systematic management of the disease. Similarly, new methods of vector control will provide cost-effective alternatives to dipping as a means of minimizing the production losses associated with the tick vectors of heartwater. An added dimension to the control of heartwater in the future will be the integration of these control methods into overall tick and tick-borne disease control strategies and into the herd-health management systems of farmers.

To achieve the goals outlined above we consider the development of all aspects of heartwater research, including vector ecology and control, epidemiology and socioeconomics, as well as vaccine development, to be essential. If any of these research areas are ignored or omitted, achieving the ultimate objective of improving the well-being of rural farmers could be considerably delayed.

ACKNOWLEDGMENTS

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Distribution, economic importance and control measures for anaplasmosis and babesiosis in South America

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INTRODUCTION

Most of Central and South America lies in the region of warm climates found between latitudes 30° N and 30° S (N-S 30°) of the equator. The N-S 30⌐ region produces almost half of the world's livestock, yet it accounts for only 30% of the world meat output (Lombardo, 1976). The continent of South America includes 12 countries with a total area of 17,707,081 km². Approximately 216 million of the estimated 600 million cattle in the N-S 30° region are found in South America alone (McDowell, 1972).

Although Latin America has twice as many cattle as the United States, the region produces less than half as much beef (McDowell, 1972). The productivity ratio is 1:4, meaning Latin America feeds four times as many cattle as the US in order to produce one tonne of beef (McDowell, 1972). This ratio must be reduced to effectively expand food supplies and improve farm income.

Ticks and tick-borne diseases have long been incriminated as major obstacles to efficient livestock production. In South America, vast areas of grazing land, although inadequate for cultivation and occupied by indigenous breeds, could yield greater quantities of animal protein were it not for constraints imposed by tick-borne diseases on the importation of improved European breeds (Smith, 1977). At present, average rates of dairy and beef production in this region are only 10–25% of acceptable values found in the northern latitudes (McDowell, 1972). Pressed by the need for economic improvement, most Latin American countries have attempted to increase the efficiency of commercial cattle operations by importing more productive breeds from countries with temperate climates. However, imported cattle are highly susceptible to tick-borne diseases and fare poorly when introduced into endemic areas.

Anaplasmosis and babesiosis may cause morbidity and mortality in susceptible cattle introduced into tick-infested areas of Mexico and Central and South America (Pino, 1981). Methods presently available for the control of these diseases rely almost entirely on the use of chemotherapeutic drugs or acaricides for the control of ticks (Smith, 1977). Undoubtedly, to implement effective control measures, renewed efforts are needed, particularly in acquiring a comprehensive understanding of tick biology and disease relationships.

FIGURE 1. Map of South America showing the eight countries from which anaplasmosis and babesiosis are described.

DISTRIBUTION AND ECONOMIC IMPORTANCE
OF ANAPLASMOSIS AND BABESIOSIS IN SOUTH AMERICA

Bovine anaplasmosis and babesiosis are widespread in most Latin American countries. The highest prevalence is found in humid, tropical and subtropical regions

(Smith, 1977). In general, anaplasmosis is more prevalent than babesiosis. *Babesia bigemina* appears to be the most prevalent *Babesia* species, although *B. bovis* seems to be more pathogenic (Lopez, 1977). Of the estimated 250 million cattle in Central and South America, approximately 175 million (70%) are in tick-infested regions between latitudes 33° North and 35° South of the equator (Lopez, 1977). Climatic conditions in those areas favour development of the one-host tick *Boophilus microplus,* the most important vector of bovine babesiosis in the region. Field studies conducted in Uruguay have demonstrated an annual output of up to six generations of *B. microplus* in certain areas (Nari *et al.,* 1979a). The distribution of ticks is restricted to high altitude, low temperature regions, especially the Andes mountain range and southern South America (Nari *et al.,* 1979a). In many areas of Central and South America and the Caribbean, *B. bigemina* and *B. bovis* are enzootically stable. The high rate of transmission of these haemoparasites results in most cattle becoming infected at a young age (Young, 1988).

Argentina. The area at risk of anaplasmosis encompasses a population of 22 million cattle in the northern region of Argentina (32° S) (Spath *et al.,* 1990). Babesiosis is restricted to areas where *B. microplus* is present (up to 30° S), representing 82 million hectares with a cattle population of 12 million head in Northeast and Northwest Argentina (Spath *et al.,* 1990). *Boophilus microplus* is found throughout the year in northeastern Argentina, with a peak incidence occurring at the end of the dry season. Other ticks found are *Amblyoma cajennense, A. neumanni* and *A. parvum* (Guglielmone *et al.,* 1990). In this region more than 80% of the cattle become seropositive to *Babesia* spp. at nine months of age (Guglielmone *et al.,* 1990). Babesiosis outbreaks are most frequently due to *B. bovis* (Guglielmone *et al.,* 1990). The Nelore breed *(Bos indicus)* presents lower infection rates with *A. marginale* and *Babesia* spp. than do the Criollo and Hereford breeds (Aguirre *et al.,* 1988). Also, Nelore cattle are known to exhibit the lowest level of *B. microplus* infestation (Aguirre *et al.,* 1988).

Uruguay. The geographic location (30–35° S) and temperate climate favours development of *B. microplus* in the northern part of the country. However, only 2.5–3.0 tick generations are observed each year (Nari *et al.,* 1979a). Approximately six million cattle are found in this region. Previous studies have also reported ticks to reside in ecological zones at the extreme south of Uruguay (34° S) (Nari *et al.,* 1979a). The southern region is only marginal for tick survival as development is limited by the low winter temperatures. The prevalence of haemoparasites on farms located at various latitudes throughout the region has been studied (Nari *et al.,* 1979a). At 31.2° S, positive seroreactors for *B. bovis* and *A. marginale* have been found in 87% and 90.4% of the farms, respectively. At 32° S, 61.2, 69.2 and 61.5% of farms were positive for *B. bovis, B. bigemina,* and *A. marginale,* respectively. In more southern latitudes (e.g. 33.5° S), no farms were positive for either *Babesia* species whereas 50% had seroreactors to *A. marginale* (Nari *et al.,* 1979b).

The cattle population in the south is affected by conditions of enzootic instability and the animals are routinely premunized (Nari *et al.,* 1979b). The negative impact of haemoparasites on cattle performance has been shown by Nari *et al.* (1979a). The authors observed that 14-month-old *A. marginale* carriers had less weight gains (47.6%) as compared with uninfected animals after a 75-day observation period. This effect was attributable to *A. marginale.* After 140 days, carriers of *Babesia* spp.

demonstrated decreased weight gain (26.1%) as compared with uninfected cattle. Livestock in Uruguay are composed exclusively of *Bos taurus* breeds. Cattle are regularly exported to neighbouring Brazil and Paraguay (Nari *et al.,* 1979b).

Paraguay. A recent serological survey reported that *A. marginale, B. bigemina* and *B. bovis* are widespread in the eastern region of Paraguay, and in much of the South and Central Chaco with rates of 92, 79 and 71%, respectively (Nari *et al.,* 1979b). An enzootically stable situation exists with regard to the three parasites over much of the country (Payne and Osorio, 1990). The livestock industry of Paraguay is based on beef production with only 3% of the six million cattle being utilized in the dairy sector. Cattle breeds are mainly Criollo *(Bos taurus* of Spanish descent) and Hereford-Zebu cross-breeds. *Boophilus microplus* is the predominant cattle tick in Paraguay with a widespread, year-long presence in cattle-rearing regions (Payne and Osorio, 1990).

Bolivia. A high prevalence of antibodies to *B. bovis* and *A. marginale* (97 and 93%, respectively) has been found in dairy cattle in the tropical, eastern region of the country (Santa Cruz) (Nicholls *et al.,* 1980). Imported *Bos taurus* suffer heavy losses when introduced into this endemic area (Nicholls *et al.,* 1980).

Brazil. Brazil occupies an area of 8.5 million km^2 in eastern South America and has a cattle population of approximately 100 million. The country consists of three large agricultural regions: *Amazon Region* is composed of four states, three territories and is characterized by the Amazon rain forest with a warm, humid climate; *Northeast Region* constitutes nine states with a characteristically semi-arid climate; and *Mid-South Region* encompasses ten states and is characterized by both tropical and temperate climates.

Most cases of cattle `tristeza' (anaplasmosis and babesiosis) occur in the southern region where most breeds are of European origin *(Bos taurus).* The prevalence of *B. bovis* in Rio Grande do Sul ranges from 46.6 to 96.1% (average 80.3%) for *B. bigemina* and 34.4 to 93.3% (average 76.2%) for *B. bovis* (Leite, 1988). In Minas Gerais, *A. marginale* prevalence has been reported to be 87.6% for Zebu cattle, 87.8% for Criollo animals, and 91.7% for Holstein cattle (Ribeiro and Reis, 1981). Babesiosis had an average prevalence of 82.5 and 79% for *B. bovis* and *B. bigemina,* respectively (Patarroyo *et al.,* 1987). In Mato Grosso do Sul, babesiosis (82.2%) is more prevalent than anaplasmosis (40.3%) (Madruga *et al.,* 1983).

Colombia. Located in the northwestern part of South America, Colombia has an area of 1.14 million km^2, of which 44 million hectares are suitable for grazing. By 1979, the cattle population was 27.1 million, the majority being for beef production. Cattle represent 87% of the country's livestock and contributes 38.7% of all agricultural output (Vizcaino, 1981). The Colombian Andes form three parallel mountain ranges that divide the country longitudinally into three major climatic zones: a hot lowland zone with an altitude below 800 m; an intermediate zone between 800 to 2,100 m; and a high zone above 2,100 m with an average annual temperature of 13 °C. The distribution of anaplasmosis and babesiosis is influenced by climate. No disease or ticks have been reported at altitudes above 2,200 m (Corrier *et al.,* 1976). Tropical and subtropical climates cover nearly 90% of the territory, providing favourable conditions for haemoparasites and their tick vectors. Twenty-three million head (85%) consisting of Criollo, Zebu and cross-bred beef cattle reside in endemic areas. Dairy cattle (approximately four million animals) of *Bos taurus* breeds (Holstein,

Brown Swiss) are found in tick-free, high altitude areas and in the subtropical intermediate zone. Serious losses have been reported in dairy farms where susceptible calves are exposed to ticks (Corrier *et al.,* 1978). The highest serological prevalence has been reported on the Caribbean coast followed by the Eastern Plains (Llanos Orientales) and the Andean region (Vizcaino, 1981). The most prevalent parasite is *A. marginale,* followed by *B. bigemina* and *B. bovis.* Except for the Caribbean coast where a situation of enzootic stability exists throughout the zone, epidemics are known to occur in other regions (Vizcaino, 1981; Gonzalez *et al.,* 1978). Further information regarding the epidemiologic characteristics of haemoparasites in Colombia is presented in Table 1.

It has long been considered that enzootic stability (abundant ticks and a high rate of parasite transmission which results in most cattle becoming infected at an early age) is desirable and economically acceptable because it precludes disease outbreaks and mortality (Dalgliesh *et al.,* 1990). However, studies (Mullenax, 1986; E.F. Gonzalez, O. Vizcaino and A. Betancourt, unpublished data) with Zebu-cross cattle and dairy cattle in stable areas of the Eastern Plains and Cauca Valley have challenged that notion. Chronic carriers of blood parasites exhibit decreased productivity as manifested by delayed conception, retarded growth, weight loss or reduced weight gains, reduced milk production, depreciated hides and lower market value (Table 2). Although it is difficult to separate production losses caused by the combined effects of anaplasmosis and babesiosis from those caused by climate, nutritional stress, ecto- and gastrointestinal parasites and other diseases, the negative impact of haemoparasitic diseases exists even under conditions of enzootic stability. An estimate of the economic losses caused by haemoparasitic diseases in the Americas is presented in Table 3.

Venezuela. Located in northern South America, Venezuela consists of a total area of 912,050 km^2. A large portion of the territory is part of the Orinoco River basin. The country can be divided into three regions: Coast and mountains, Orinoco plains and Guyana region. Only the first two regions are relevant for livestock production. The cattle population is 10.53 million; 38.4% (4.05 million) are for dairy and 61.6% (6.48 million) for beef production. In the first region, the Colombian Oriental mountain range merges into two separate ranges named Perija and Merida. These surround Maracaibo Lake and the Gulf of Maracaibo. To the east and parallel to the littoral lie the Caribbean mountain range. In this region the climate varies from tropical to temperate to cool according to the altitude. Most dairy farms are located in this region with 85% of milk produced in the states of Zulia, Lara, Falcón, Táchira, Mérida and Trujillo (Toro *et al.,* 1992). The most common breeds are *Bos taurus* (Holstein, Brown Swiss) and Criollo crosses (Carora breed) or Brahman (Siboney breed). An important contingent of pure-bred Holstein cattle are imported annually from the USA, New Zealand and Canada (Toro *et al.,* 1992).

Beef farms are concentrated in the Venezuelan plains located south and southeast of the Mérida and Caribbean mountain ranges west of the Orinoco River. This area consists of savannah grasslands where cattle operations are extensive in nature with open range-type management. Predominant cattle breeds are Zebu, Criollo and Zebu-Criollo crosses. The principal beef-producing states are Apure, Barinas, Portuguesa, Cojedes, Guárico, Anzoátegui and Monagas (Toro *et al.,* 1992). Sero-epidemiological surveys conducted in dairy farms of the midwestern region of

TABLE 1. Epidemiological characteristics of haemoparasites in important cattle regions of Colombia (1979).

Region	Herd (mill.) Breed	Hectares (mill) Type explot.	Seroprevalence			Patency (%)				Mortality %	Epid Status
			A. marginale	*B. bigemina*	*B. bovis*	*A. marginale*	*B. bigemina*	*B. bovis*	*T. vivax*		
CARIBBEAN	10.9	4.7									
North Coast	Zebu	Beef	83–93	57	—						
	Criollo	Beef									
Baranquilla	Cross-bred	Beef	57.4	50	38.1	92.3	7.6	7.6	23	2.2–7.7	Stable
Valledupar			77.8	67.1	63.1	l00	15.4	7.6	15.4		
			(23.2)*								
LLANOS ORIENT.	3.4		71–74	62–64	67.3						
Piedemonte	Z/Cr/Cb	Beef	62.4	40.1	41.6	63.6	9	9	27.2		Stable
Sabana			(24.2)*								Unstable
ANDEAN	3.47	Dairy	45	46.6	26.0					3.7–18	Unstable
	Bos taurus										
Valle del Cauca	6.81	Double	73	62.5	65.4						
	Z/Cr/Cb	Beef	88.1	87.4	44.4						
La Dorada			67 (39)*		51.7						
Manizales			34.2	27.8	26.9	72.7	9	9	0		
Bucaramanga			69	50.8	53.2	70	10	10	10		
			(15.7)*								

* Tailed *A. marginale*
Adapted from Vizcaino, O. 1981. ICA-IICA Bull. No. 25l.
T. vivax = Trypanosoma vivax

Venezuela have found more cattle seropositive to *B. bigemina* (78.2%) than to *B. bovis* (38.8%). The prevalence of antibodies to anaplasmosis was reported to be 57.7% with an overall *A. marginale* infection rate of 21.1% (James *et al.,* 1985). Venezuela offers a wide range of epidemiological conditions because highly susceptible pure-bred cattle are imported regularly. The diversity of management practices, geographic characteristics, farm size, etc. produces a milieu that provides varying degrees of enzootic stability for these infections (Montenegro-James *et al.,* 1989). Seroprevalence studies carried out in major cattle regions of Venezuela indicated that the average prevalence for *B. bovis* was 33.1%, ranging from 6.3 to 79.4%, with the mean *B. bigemina* prevalence of 47.1% (range 2.2 to 89.5%) (Montenegro-James *et al.,* 1989). The average prevalence for *A. marginale* was 51.3% with a range of 22.2 to 88.1% (Toro *et al.,* 1992). Seventeen farms that were involved in the sero-epidemiological studies demonstrated relatively low seroprevalence rates of bovine babesiosis (range 2.2 to 14.0%) for farms with systematic tick dipping and various degrees of cattle confinement, compared to 96% prevalence when sporadic dipping and grazing were practised (Figure 2) (Montenegro-James *et al.,* 1989). In Zulia State and the various Andean states where dairy farms predominate, the highest prevalence was found for *A. marginale* followed by *B. bigemina* and *B. bovis,* whereas in beef-producing regions (Plains) the prevalence was similar for each of the three parasites (Figure 3).
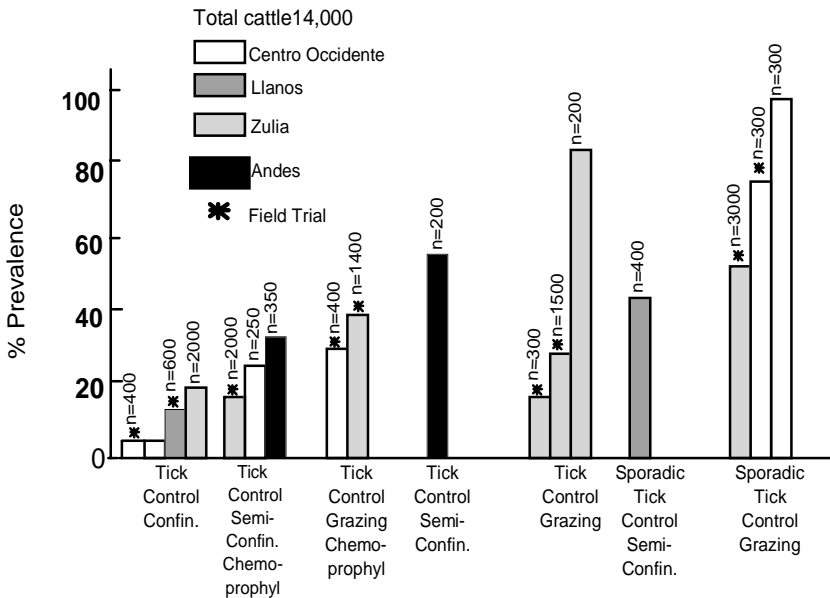

FIGURE 2. Serological prevalence of bovine babesiosis in 17 dairy farms (various locations throughout Venezuela) managed under varying conditions for parasite and tick control. Reprinted with permission from *Veterinary Protozoan and Hemoparasite Vaccines,* CRC Press, Inc. (Montenegro-James *et al.,* 1989).

TABLE 2. Summary of losses in a 100-head herd due to stable enzootic anaplasmosis and babesiosis—Colombian llanos (Mullenax, 1986).

	$
Calves and calfhood losses	275.05
Milk production losses	100.80
Failure of weight gain in fattening steers	36.15
Failure of weight gain in fattening females	6.89
Failure of weight gain in stockers and feeders	55.35
Death loss in bulls	93.50
Total/100-head herd with typical population distribution	567.74
Total estimated per-animal loss in a 100-head herd	5.68/head
Total estimated per-head treatment cost	1.87
Total per-head cost of loss and treatment distributed over total population	7.55/head

Valle del Cauca 1975 (Gonzalez *et al.,* unpublished data).
Losses in dairy cows (chronic carriers of anaplasmosis and babesiosis).
Average—605 l milk/year than non-affected dairies.
Higher incidence of infertility and abortions.

Guyana. Babesia infections in cattle are widely distributed with a high prevalence found in all ecologically defined regions of the country, i.e. coast, forest and savanna. The seroprevalence for *B. bigemina* has been reported at 80.1% (range 73.1 to 100%), whereas the prevalence for *B. bovis* was 61.4% (range 45.2 to 88.2%) (Applewhaite *et al.,* 1981).

CONTROL MEASURES FOR ANAPLASMOSIS AND BABESIOSIS IN SOUTH AMERICA

Control measures employed in most South American countries for anaplasmosis and babesiosis essentially rely on chemotherapy, use of acaricides to control *B. microplus* and, to a lesser degree, on immunization methods. In general these measures are expensive, time-consuming and in many cases of limited success.

Chemotherapy. For babesiosis, diamidine derivatives (Berenil®, Ganaseg®, Pirobenz®) are frequently used. Imidocarb dipropionate (Imizol®) is also recommended for chemoprophylaxis (Anon, 1988). For anaplasmosis, the drugs of choice are long-acting tetracyclines and imidocarb, although the latter has not always proven effective (Adams and Todovoric, 1974). Because of the need for higher dosages and longer treatment regimens for anaplasmosis, the use of drugs is generally limited to control outbreaks and not to sterilize carriers.

Tick Control. Initially, ambitious aims towards eradication of ticks induced several countries to engage in expensive and unsuccessful national campaigns for tick control. Mexico, Cuba, Costa Rica, Colombia, Uruguay and Argentina developed such programs. Problems with cost, acaricide resistance, infrastructure and maintenance were important deterrents. Currently in most countries, the approach has been
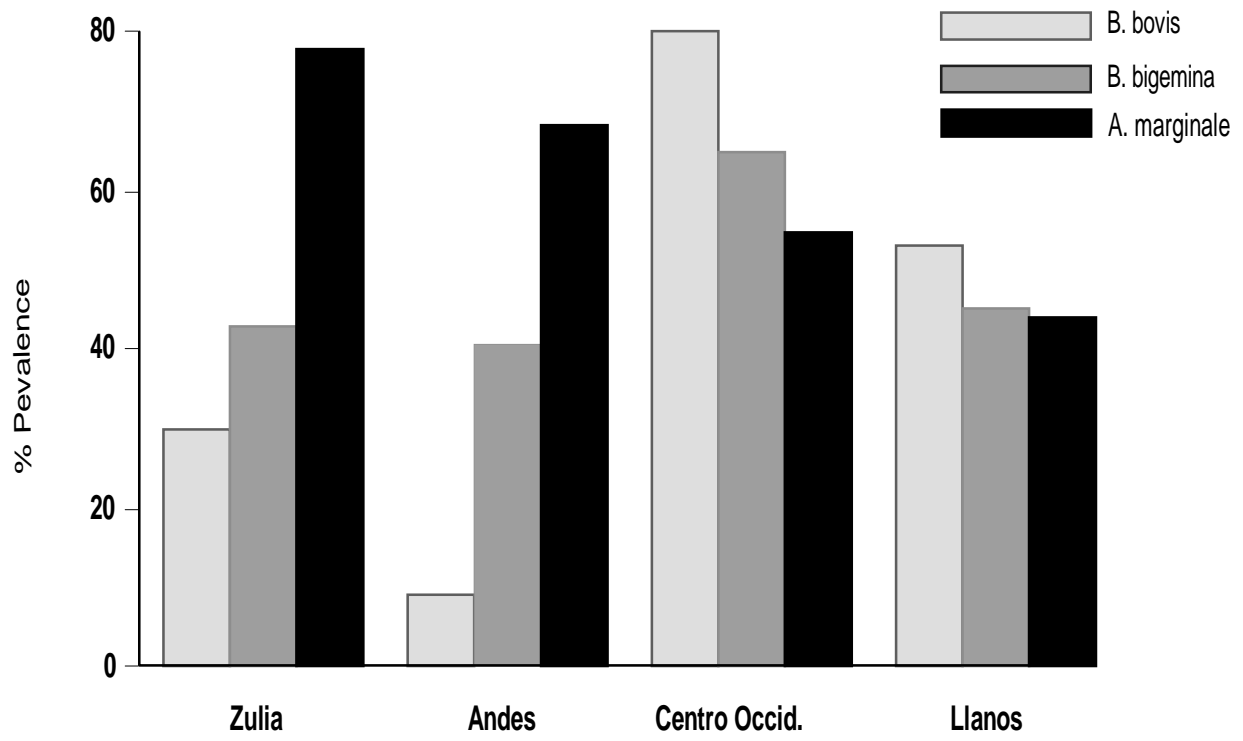
FIGURE 3. The prevalence of antibodies to *Anaplasma marginale* and *Babesia bovis* in cattle in different regions in Venezuela.

TABLE 3. Estimate of economic losses associated with anaplasmosis and babesiosis in the Americas.

Country/Region	Cattle No. (millions)	Pathogen(s) Enzootic Status	Total cost ($ million/yr)	Average and cost/head/yr	Reference
		Anaplasma marginale			
USA	118.0	enzootic-nonenzootic	100	0.85	McCallon, 1973
California	4.0	non-enzootic	5.2–11.0	1.33	Goodger *et al.,* 1979
Texas	12.8	non-enzootic	9.0	0.70	Alderink and Dietrich, 1983
		Anaplasma and *Babesia*			
Mexico	35.0	enzootic-nonenzootic	287	8.2	Mullenax, 1986
Colombia	27.1	enzootic-nonenzootic			
(Llanos)	22.0	stable enzootic	133	3.2	Mullenax, 1986
Brazil	99.5	enzootic-nonenzootic	—	—	
R. do Sul	9.89	enzootic-nonenzootic	99	9.9	Madruga, 1984
Argentina (North)	12.0	enzootic-nonenzootic	120		Spath *et al.*, 1990
Venezuela	10.0	enzootic-nonenzootic	23.3[*]		Toro-Benitez, 1985
Central America	58.0	enzootic-nonenzootic	850	3.12	Lombardo, 1976
South America	215.6	enzootic-nonenzootic	1365–1638	5–6	Anon, 1987

[*]Mortality only

one of discretional suppression in which the livestock owner makes a decision to reduce tick populations. The most common acaricides are organophosphorous compounds, formamidine and pyrethroids (A. Guglielmone, personal communication).

Immunization. Based on the success achieved in Australia with the use of *A. centrale* and attenuated *B. bovis* and *B. bigemina* as vaccines against anaplasmosis and babesiosis, adaptation of these methods was attempted in the 1970s in Bolivia, Venezuela, Colombia, Uruguay and Argentina. The latter two countries were true pioneers in the use of premunization techniques. The initial work of Prof. Jose Ligneres in Buenos Aires at the beginning of the century was followed by that of Prof. Miguel C. Rubino in Uruguay (Muskus and Leon, 1965). Possibly the lack of adequate technology, infrastructure and the availability of material have accounted for the limited effectiveness of those methods (Callow, 1974). Premunization utilizing local isolates has produced severe reactions that necessitate chemotherapy and veterinary surveillance in order to control disease (Todovoric and Tellez, 1975; Todovoric *et al.,* 1978; Corrier *et al.,* 1980). In Brazil premunization is employed without official control or veterinary supervision. Limited amounts of attenuated *B. bovis* and *B. bigemina* vaccines are produced in a few veterinary research institutions (J. Patarroyo, personal communication).

In Venezuela premunization was officially applied in all imported cattle by the Institute of Climatization and Premunization until the 1950s (Muskus and Leon, 1965). Recently, an inactivated culture-derived *B. bovis-B. bigemina* vaccine has been produced at the Veterinary Research Institute in Maracay and used to vaccinate approximately 15,000 cattle with satisfactory results (Montenegro-James, 1989;

Montenegro-James *et al.* 1992a). More recently, an inactivated *A. marginale* vaccine consisting of isolated initial bodies, either administered alone (Montenegro-James *et al.,* 1991) or simultaneously with the *Babesia* vaccine, has been evaluated. Promising results have been obtained in both laboratory and field trials (Montenegro-James *et al.,* 1992b).

Uruguay has utilized an attenuated *B. bovis* and *B. bigemina* vaccine derived from local isolates and *A. centrale* (from Israel) for anaplasmosis since 1941. Premunition procedures are adequately supervised. Cattle exported to Paraguay and Brazil are regularly vaccinated. However, Payne *et al.* (1990) have reported the failure of *A. centrale* to protect cattle against *A. marginale* challenge. The same authors also indicated that although the *Babesia* vaccine is generally satisfactory, up to 38% of vaccinated cattle may require treatment.

In Argentina, limited use of live attenuated *Babesia* vaccines and *A. centrale* has proved satisfactory (Anziani *et al.,* 1987; Guglielmone, 1980). A recent cost-benefit analysis indicated that there is a considerable demand in Argentina for an effective vaccine(s) against anaplasmosis and babesiosis (Spath *et al.,* 1990).

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Anaplasmosis, babesiosis and cowdriosis in the West African sub-region: distribution, economic importance and control measures

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INTRODUCTION

The West African sub-region consists of Nigeria, Ghana, Cote D'Ivoire, Benin Republic, Togo, Cameroon, Liberia, Sierra Leone, Guinea, Senegal, Niger, Chad, Mali, The Gambia, Burkina Faso, Equitorial Guinea and Mauritania. The region covers the following vegetational zones: Sahel, Sudan, Northern and Southern Guinea savanna zones, derived savanna, rain forest and mangrove forest. Anaplasmosis, babesiosis and cowdriosis (heartwater) constitute the major tick-borne diseases of domestic ruminants in the region where they are endemic. These diseases cause considerable economic losses through death, decreased meat and milk production, unthriftness, decreased draught power together with the cost of control measures. They are also a major constraint to improved breed and livestock development. This presentation will draw, to some extent, on recent studies carried out under the EEC-sponsored ticks and tick-borne diseases project executed by the Ahmadu Bello University, Zaria, and the Royal University of Utrecht, the Netherlands.

DISTRIBUTION

Anaplasmosis

Two species of the genus *Anaplasma, A. marginale* and *A. centrale,* have been recorded as occurring in Africa, although doubts have been expressed about *A. centrale* occurring as a distinct species from *A. marginale.* Anaplasmosis occurs in the whole of the West African sub-region. Ticks and biting flies occur widely throughout the whole region, but it is difficult to determine the relative importance of ticks or flies in the transmission and maintenance of the diseases. It is reasonable to assume, based on reports of the disease's occurrence elsewhere, that both ticks and biting flies are likely to be involved.

Serological studies carried out under the EEC-sponsored ticks and tick-borne diseases project in different ecological zones in Nigeria showed that the prevalence
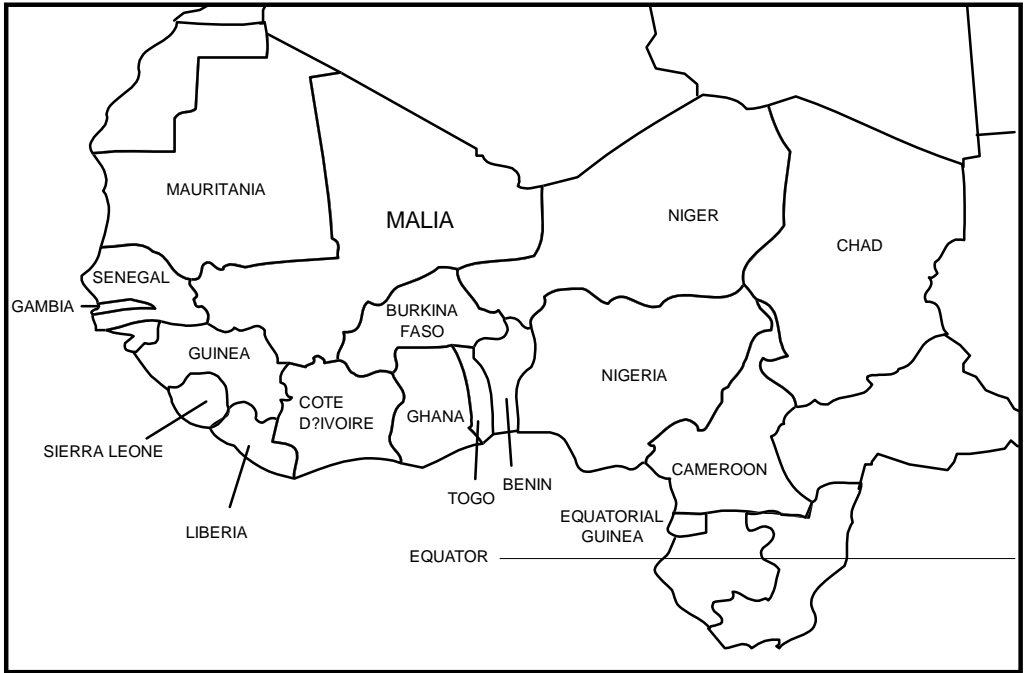
FIGURE 1. The West African sub-region.

of antibodies against *A. marginale* in cattle in the main ecological zones was 0–40%, irrespective of whether it was during the wet or the rainy season (Table 1) and that the prevalence of *A. marginale* was also generally low using thin blood smears.

Babesiosis

Two species of *Babesia, B. bigemina* and *B. bovis,* are reported to occur in cattle in the sub-region (Leeflang and Ilemobade, 1977). The major tick genera found in the sub-region are *Boophilus, Amblyomma, Hyalomma* and *Rhipicephalus.* Only boophilid ticks are known to transmit *Babesia* species in this region. The three boophilid species reported in Nigeria and which have been reported to occur in some parts of the sub-region are *Boophilus decoloratus, Boophilus geigyi* and *Boophilus annulatus* (Mohammed, 1974). *Boophilus decoloratus* is more abundant in the Sudan and Northern and Southern Guinea savanna zones than the other two species. *Boophilus geigyi* seems to be as abundant as *Boophilus decoloratus* in the wet forest zones, a reason why Dipeolu (1975) argued that it may be the main transmitter of *B. bovis.* It has, however, proved difficult to establish the tick responsible for transmitting *B. bovis* in the sub-region. The widespread distribution of *B. bovis* in the

TABLE 1. Prevalence of antibodies against *Anaplasma marginale* in cattle in Nigeria, using IFAT on samples from ranches in the main ecological zones.

Season	Dry season (1985/86)		Wet season (1986)		Wet season (1987)	
Age (years)	<3	≥3	<3	≥3	<3	≥3
Sudan zone						
Gidan Jaja	11% (18)	17% (47)	5% (20)	37% (38)	6% (32)	29% (51)
Bunkure	12% (34)	27% (48)	4% (19)	51% (35)	19% (27)	31% (65)
Gombole/Gajuba	15% (41)	26% (38)	15% (20)	20% (49)	—	—
Northern Guinea Savanna zone						
Shika	0% (11)	7.5% (67)	50% (30)	46.5% (71)	26.5% (34)	42% (53)
Vom	12% (26)	40% (52)	45% (31)	8.6% (35)	16% (19)	21% (42)
Southern Guinea Savanna zone						
Mokwa	0% (32)	0% (69)	0% (30)	0% (76)	5.7% (35)	0% (65)
Raav	—	—	0% (29)	0% (28)	0% (28)	0% (35)
Forest zone						
Akunnu	—	—	0% (11)	0% (39)	0% (40)	0% (30)
Fashola	14% (14)	6.9% (87)	6.6% (30)	2.8% (35)	6.6% (30)	1.7% (56)
Mountain zone						
Obudu	22% (63)	9.4% (32)	7.9% (27)	2.7 (36)	—	—
Mambilla	—	4.6% (87)	3.8% (26)	0% (38)	—	—

Actual figures in brackets.
Source: EEC-sponsored ABU/RUU ticks and tick-borne diseases project.

brain smears of slaughtered cattle in Nigeria, and the scanty population of *Boophilus annulatus* and *Boophilus geigyi* in the areas of highest cattle concentration, however, suggest that the identification of the vector for *B. bovis* in Nigeria needs further investigation. It may well be that *Boophilus decoloratus* is involved.

Cowdriosis

Cowdriosis (heartwater) is widespread in the sub-region except for the very dry ecological zones like Sahel, where *Amblyomma* spp. ticks are very rare. It has been established that the disease is transmitted exclusively by ticks of the genus *Amblyomma* and, since these ticks are probably the most widespread in the sub-region, it is not surprising that heartwater is also widespread.

ECONOMIC IMPORTANCE

Tick-borne diseases are major limiting factors to successful animal production in the sub-region. They are the cause, in cattle, of high morbidity and mortality, decreased meat and milk production, and loss of draught power and manure. They are also an impediment to the upgrading of indigenous breeds of cattle, sheep and goats and to the introduction of more productive, exotic breeds.

TABLE 2. Prevalence of antibodies against *Babesia bigemina* in cattle in Nigeria using IFAT on samples from ranches in the main ecological zones.

Season	Dry season (1985/86)		Wet season (1986)		Wet season (1987)	
Age (years)	<3	≥3	<3	≥3	<3	≥3
Sudan savanna						
Gidan Jaja	50%(26)	80%(70)	3.4%(18)	3.3%(42)	3%(33)	7.6%(52)
Bunkure	18.9%(37)	25%(60)	0%(24)	2.1%(48)	28.5%(28)	25.8%(62)
Gombole/Gujuba	33.3%(51)					
Northern Guinea						
Vom	43%(30)	67.6%(65)	30%(30)	45%(42)	18.7%(16)	14%(49)
Shika	6%(15)	40%(80)	72.4%(29)	62.5(80)	35%(34	16.9%(63)
Southern Guinea						
Raav	—	—	0%(12)	0%(28)	3.5%(28)	0%(35)
Mokwa	12.5%(32)	33%(69)	6.6%(32)	5.2%(76)	0%(32)	3%(65)
Forest Zone						
Akunnu	—	—	18.1%(11)	30.7%(39)	15%(40)	46.6%(30)
Fashola	14.2%(14)	32.2%(87)	7.1%(28)	57.1%(35)	6.6%(30)	12.5%(56)

Source: EEC-sponsored ABUU/RUU ticks and tick-borne diseases project.
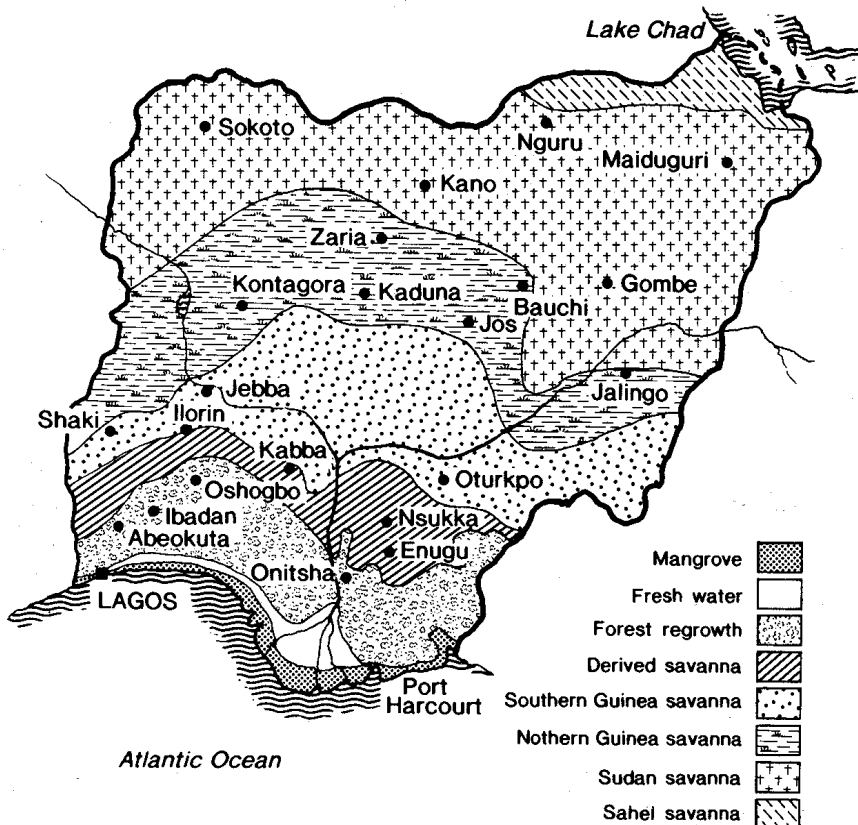
FIGURE 2. Map of Nigeria showing eight vegetation zones.

CONTROL MEASURES

There are three major approaches to the control of tick-borne diseases of livestock in the West African sub-region.

Vector control ensures that the incidence of the disease is kept within manageable limits. The two commonly used methods are hand de-ticking and the application of acaricides. The escalating costs of labour and acaricides make the use of acaricides a less attractive option. Fortunately the problem of acaricide resistance in ticks has not, as far as can be established, arisen and if costs can be managed, this method is likely to remain a practical control measure for the foreseeable future.

Chemotherapy, when used under veterinary supervision, allows, in conjunction with acaricidal control, for some measure of pre-immunity or immunity to be established against the diseases. Drugs can be used during the period of initial exposure in a method known as chemo-immunization. However the method can be expensive and relies on challenge occurring during the period of drug cover which may create problems under a pastoral system. Chemotherapy of sick animals is fairly widely used.

Immunization using live blood vaccines has been used only under experimental conditions.

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Bovine babesiosis and anaplasmosis: distribution, economic importance and control measures in Southeast Asia

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INTRODUCTION

Southeast Asia is a large and not clearly defined area of the world and for the purposes of this paper `Southeast Asia' describes the 12 countries listed in Table 1. The single host cattle tick, *Boophilus microplus,* has been identified in all these countries and the haemoparasites *Babesia bovis, B. bigemina* and *Anaplasma marginale* it transmits are endemic in the cattle populations (Brockelman, 1990). Other ticks of the genera *Boophilus* and *Rhipicephalus* may also transmit one or more of the haemoparasites to cattle complicating the epidemiology (Brockelman, 1990).

Few detailed studies have been made of the distribution, economic importance and control of babesiosis and anaplasmosis in any Southeast Asian country. The Australian Centre for International Agricultural Research (ACIAR) established a project in Sri Lanka on `Control of tick-borne diseases of ruminants in Sri Lanka with particular reference to babesiosis and anaplasmosis'. The Queensland Department of Primary Industries (QDPI) was the commissioned organization and after a favourable review at completion in 1990, the project's achievements were assessed by an independent economist. Sri Lanka can be considered a model for Asia because it encompasses a variety of cultures, topography and climate and the observations made during the course of this project may serve as examples of what could occur in the other countries.

DISTRIBUTION OF BABESIOSIS AND ANAPLASMOSIS IN SOUTHEAST ASIA

The predominantly *Bos indicus* indigenous cattle in Southeast Asia coexist in relative harmony with the haemoparasites and their vector with little evidence of clinical babesiosis and anaplasmosis (Dalgliesh *et al.,* 1990). Livestock improvement programs in the region during the last decade have resulted in a steady increase in high yielding *Bos taurus* cattle introduced from cool temperate climates. The combined stresses of shipment, change of management, introduction to a hot, humid climate and exposure to a variety of diseases for the first time have resulted in spectacular losses

TABLE 1. Countries of Southeast Asia where *Boophilus microplus* ticks have been identified[*].

Brunei	Indonesia	Phillipines
Burma	Malaysia	Sri Lanka
China	Pakistan	Taiwan
India	Papua New Guinea	Thailand

[*]Hoogstraal, 1956; D.H. Kemp, personal communication.
 Countries underlined recognize babesiosis and anaplasmosis as threats to imported cattle.

amongst these European breed cattle. Tick-borne diseases, including babesiosis and anaplasmosis, are significant contributors to these losses (Dalgliesh *et al.,* 1990). The countries underlined in Table 1 have purchased vaccine against babesiosis and anaplasmosis, requested assistance in the diagnosis of the diseases or have requested the transfer of technology to produce the vaccines themselves. These countries consider babesiosis and anaplasmosis to be a major threat to local and imported susceptible cattle.

Distribution in Sri Lanka

The first objective of the ACIAR project in Sri Lanka consisted of a serological survey of cattle in widely separated localities to identify the approximate distribution of *B. bovis* and *Anaplasma.* Antibodies were detected in all localities tested and indicated that climatic difference had no substantial effect on the distribution (Jorgensen *et al.,* 1992). A second objective of the project was to assess the risk of disease outbreaks occurring in the locations tested. Risk assessment for *B. bovis* can be based on the seroprevalence of antibodies in groups of cattle over nine months of age (Mahoney and Ross, 1972). At this age immunity conferred by colostral antibodies and calfhood resistance to both *Babesia* and *Anaplasma* has waned and cattle without antibodies by this age are potentially at risk to the diseases. The seroprevalence for *B. bovis* in all Sri Lankan localities below 1,200 metres, where the cattle are predominantly *Bos indicus,* was consistently in excess of 90%. Cattle located above 1,200 metres in the hill country showed a seroprevalence ranging from 13 to 100%. The comparatively low proportion of cattle with antibodies to *B. bovis* in certain locations in the hill country suggested a high susceptibility to infection and clinical disease. The risk is compounded by the fact that this group comprises *Bos taurus* breeds, genetically the most susceptible to *Babesia* infections (Jorgensen *et al.,* 1992).
  The low seroprevalence for *B. bovis* in some of the localities above 1,200 metres was due to the management practice, common to many Sri Lankan hill country farms of maintaining calves indoors away from tick-infested pastures until nine months of age. Deaths due to babesiosis in recently released cattle have been reported in recent years on many of the properties in the hill country (Jorgensen *et al.,* 1992). The seroprevalence for *Anaplasma* varied considerably (18–90%) amongst localities and

did not correlate with climatic zones. Seroprevalence varied greatly even at two localities very close together (Jorgensen *et al.,* 1992) and further investigation of *Anaplasma* transmission is required.

CONTROL IN SOUTHEAST ASIA

Clinical cases of babesiosis and anaplasmosis are seldom diagnosed in indigenous *Bos indicus* in Southeast Asia (Brockelman, 1990). However, spectacular losses do occur such as when unvaccinated New Zealand cattle were imported into Indonesia where the haemoparasites are endemic (R.C. Payne, unpublished data).

Control of babesiosis and anaplasmosis in Sri Lanka

In addition to defining the seroprevalence, a further objective of the ACIAR project was the development of a production facility for live babesiosis and anaplasmosis vaccine. The serological surveys had shown that the cattle most at risk were susceptible *Bos taurus* breeds maintained in the cooler hill country which is relatively free of other diseases such as foot and mouth and brucellosis. The Sri Lankan authorities had earlier recognized the risk of the diseases and introduced the carrier/donor vaccination system in the late 1940s to reduce losses when *Bos taurus* cattle were first imported to upgrade milk production. The carrier donor system relies on chronically infected animals to supply infected blood for vaccination of susceptible cattle. Problems that were encountered with this form of vaccination were similar to those which occurred in Australia. These included extremely variable infection rates with the cattle not always being protected, unpredictability of reactions and the transfer of other field strains and blood parasites to cattle being vaccinated. Increasing numbers of *Bos taurus* animals imported in the 1970s and 80s highlighted the deficiencies of the carrier donor system. The proposed vaccine production facility in Sri Lanka would use similar procedures to those developed in Queensland. Use of infected blood from splenectomized donor calves in this production method ensures infectivity of the vaccine, minimizes transfer of pathogenic organisms and minimizes vaccine reactions by using attenuated strains (Callow and Dalgliesh, 1980).

Initially the Sri Lankan authorities wished to attenuate a local strain of *B. bovis* rather than release the Australian vaccine strain. Suitable laboratory facilities for tick-free calf accommodation were identified and attenuation of a local *B. bovis* isolate by serial syringe passage in splenectomized calves was initiated and continued for more than 30 passages (Weilgama *et al.,* 1989). The virulence and protection of the Sri Lankan and Australian *B. bovis* vaccine strains were compared in 37 susceptible *Bos taurus* bulls imported from Victoria, Australia. Animals vaccinated with the Australian *B. bovis* vaccine strain experienced mild reactions while those vaccinated with the Sri Lankan strain experienced unacceptably severe reactions (Weilgama *et al.,* 1989). Both strains protected well against subsequent field challenge when the animals were distributed to centres in the mid and hill country.

Cattle vaccinated with attenuated strains of *B. bovis* and *B. bigemina* can have significant cross protection against subsequent challenge by vector-transmitted heterolo-

gous field strains. The Australian *B. bovis* and *B. bigemina* vaccine strains have now been shown to successfully protect susceptible *Bos taurus* cattle against challenge by pathogenic strains in Bolivia, South Africa (Dalgliesh *et al.,* 1990) and Malawi (J. Lawrence and J. Malika, personal communication). There is also evidence that the Australian vaccine strains protect against field strains in Malaysia, Thailand and Trinidad (A.J. de Vos and co-workers, 1990, unpublished observations). The chilled vaccine now produced in Sri Lanka is made using the Australian *B. bovis* vaccine strain (K). Like the Australian product, it has a shelf life of six days if stored at 4 °C. This short shelf life does not allow testing for pathogenic organisms after production nor does it allow easy dissemination to remote areas prior to the expiry date. Further development has resulted in the production of a new frozen vaccine stored in liquid nitrogen that can be kept for at least five years. The new product is manufactured as a dilutable concentrate that allows comprehensive testing for infectivity, efficacy and confirmation of the absence of pathogenic organisms (Jorgensen *et al.,* 1989; Dalgliesh *et al.,* 1990). The advantages of the new frozen vaccine are reviewed in detail by Dalgliesh *et al.* (1990).

Drug therapy in Sri Lanka

Acute babesiosis and anaplasmosis may be controlled by treatment. However, vaccination is the only viable long-term strategy for the protection of susceptible cattle. The only readily available drug for treatment of acute babesiosis in Sri Lanka is diminazene aceturate (Berenil, Hoechst). Oxytetracycline (Terramycin, Pfizer) is available for treatment of anaplasmosis. Another drug, imidocarb dipropionate (Imizol, Pitman Moore), an effective treatment for both babesiosis and anaplasmosis, may become available; however its cost relative to diminazene makes widespread use unlikely in Sri Lanka. Imidocarb has a prophylactic effect for babesiosis for six weeks at a dose rate of 2 ml/100 kg, but it is not satisfactory for long-term use due to toxic effects with prolonged use (A.J. de Vos and W.K. Jorgensen, unpublished observations).

   Most female breeding cattle shipped to Sri Lanka and other countries of Southeast Asia are pregnant (B. Barker, personal communication). In the experience of the staff at the Tick Fever Research Centre (TFRC), there is much greater risk of abortion for cows in the third trimester of pregnancy if they develop acute babesiosis. Severe reactions may occur even with attenuated vaccine strains and further research is under way at TFRC to develop a vaccination protocol that minimizes risk for pregnant cows (Jorgensen *et al.,* 1992). Until a suitable protocol for vaccination of pregnant animals is available, the prophylactic use of imidocarb until calving may be a viable option for protecting susceptible cows entering Southeast Asian countries. After calving it is recommended that the animals be vaccinated no sooner than eight weeks after the last imidocarb treatment and maintained tick free until vaccination takes effect.

ECONOMIC IMPORTANCE IN SOUTHEAST ASIA

Brockelman (1990) reviewed the economic importance of babesiosis and anaplasmosis in Southeast Asia and observed that, of an estimated 337 million

cattle in Asian countries, perhaps 2–20% are affected by the diseases. In Sri Lanka and other Asian countries, the cattle most in danger from babesiosis and anaplasmosis are those that represent the greatest economic investment such as high quality breeding stock imported to increase milk and meat production (Brockelman, 1990; Dalgliesh *et al.,* 1990; Jorgensen *et al.,* 1992). The major exporters of bovine breeding stock to Southeast Asian countries are USA, Canada, Australia and New Zealand (B. Barker, personal communication). *Babesia bovis* and *B. bigemina* are not present in USA, Canada, New Zealand and four of the seven Australian states. *Anaplasma marginale* occurs in USA and Australia but not all cattle are protected by previous infections or vaccination. Clearly, many cattle exported from these countries will be susceptible to babesiosis or anaplasmosis unless vaccinated. The Australian component of the live cattle trade to Southeast Asia is shown in Table 2. Assuming an imported value of US$800 for a breeding cow and more for bulls, these figures represent a considerable investment for the importing country. Should the animals die, the economic loss is not only the purchase price but the potential contribution of that animal to the genetic improvement of the national herd.

TABLE 2. Live cattle exports from Australia to Southeast Asia.

Destination	Purpose of cattle			Total
	Breeding	Fattening	Slaughter	
Malaysia	443	11,887	9,846	22,176
Brunei	—	103	6,712	6,712
Thailand	2,648	—	—	2,648
Phillipines	—	578	19,118	20,544
Indonesia	5,692	1,200	—	6,892

Data from Australian Meat and Livestock Corporation showing the means for the period 1988–1990.

Economic importance in Sri Lanka

The economic importance of babesiosis and anaplasmosis to a Southeast Asian country may again be demonstrated using Sri Lanka as an example. ACIAR commissioned an independent economist to assess the value of the benefits that had been developed during the QDPI project on tick-borne diseases of ruminants (Chudleigh, 1991). Since the Veterinary Research Institute in Sri Lanka adopted a new system of vaccine production using splenectomized donor calves, they can provide a vaccine which is more effective and less dangerous to the recipient animal than the carrier donor-derived vaccine previously used. The new frozen vaccine developed during the project makes the best use of this production system, cryopreserving it as a dilutable concentrate which can be stored for at least five years (Dalgliesh *et al.,* 1990). The most important benefit of the project to Sri Lanka will be that valuable, imported

cattle will be more fully protected by the new vaccine (Chudleigh, 1991). Approximately 100 *Bos taurus* animals will be imported into Sri Lanka annually as part of a genetic upgrading scheme (Chudleigh, 1991). An estimated 25% mortality could be expected amongst susceptible animals entering an area endemic for babesiosis and anaplasmosis. Chudleigh (1991) calculated that a saving of US$18,000 per annum could be directly attributed to provision of safe effective babesiosis and anaplasmosis vaccines. Other less definable benefits to Sri Lanka include reduced mortality and increased productivity amongst the F1 generation of breeding stock in the enzootically unstable hill country. These first generation cattle have an estimated value of five times that of the indigenous *Bos indicus* breeds (Chudleigh, 1991). The primary function of the six large government farms holding these cattle is supplying most of the island's milk. However, they also sell excess animals to other farms and small holders at nominal prices, further increasing productivity. Prior to the vaccination programs assisted by the ACIAR project, Chudleigh (1991) calculated the annual cost of mortality on these farms from babesiosis and anaplasmosis to be US$40,000 per annum. Morbidity effects due to babesiosis and anaplasmosis are more difficult to determine. Affected animals can develop severe anaemia and liver damage usually losing weight and condition. Acute disease can affect milk production and a basal body temperature rise above 40.5 °C during severe reactions can cause abortion in late pregnancy and temporary loss of fertility in bulls.

As the proportion of vaccinated cattle in the hill country increases, the number of field cases will decrease, minimizing expensive chemotherapy. Treatments for babesiosis and anaplasmosis in Sri Lanka cost 1.1 and 4 US cents respectively. The government, however, subsidizes drug costs (Chudleigh, 1991). Chudleigh (1991) estimates that US$220 was spent annually on the treatment of babesiosis and anaplasmosis on government farms prior to use of a safe, effective vaccine. Manufacturing babesiosis and anaplasmosis vaccines in Sri Lanka minimizes labour costs, making the cost per dose more affordable to small land owners. Although their weekly income may be only US$7, they are now able to take advantage of buying higher yielding *Bos taurus* cross-breed animals without fear of them dying from babesiosis and anaplasmosis.

CONCLUSION

In Southeast Asia indigenous *Bos indicus* cattle are usually located in babesiosis and anaplasmosis endemic areas where they are infected early in life and become resistant to the disease. The cattle at greatest risk are susceptible *Bos taurus* breeds imported to improve meat and milk production. The best protection available at present is a single vaccination with living vaccines against the haemoparasites from which susceptible cattle acquire a long-lasting immunity.

ACKNOWLEDGEMENT

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DIAGNOSIS

Development of diagnostic reagents for anaplasmosis and babesiosis

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INTRODUCTION

Sensitive and specific assays for detection of microbial pathogens provide critical tools for identification of sick animals requiring prompt, appropriate therapy and for disease prevention by regulation of animal movement. In addition, defined assays are required for understanding epidemiology including the parameters that influence herd immunity. Although diagnosis of acute *Anaplasma* and *Babesia* infections in cattle based on clinical signs and identification of intraerythrocytic organisms has been used for most of this century, epidemiological research has been constrained by the lack of diagnostic assays suitable for large numbers of carrier cattle and arthropod vectors. Development of improved diagnostic assays provides new opportunities for haemoparasitic disease research. In this brief review, I will focus on recent developments and current needs in diagnostic assays for *Anaplasma marginale, Babesia bigemina* and *B. bovis.*

DIRECT ORGANISM DETECTION

Acute infection in cattle. Direct detection of intraerythrocytic organisms provides identification of animals with active infection and eliminates variables that confound serological diagnosis such as colostral antibodies, post-vaccination antibodies, or antibodies in animals spontaneously or chemotherapeutically cleared of infection. Wright-Giemsa stained blood smears from cattle with clinical signs remains the most commonly used method to confirm diagnosis of anaplasmosis or babesiosis. For *B. bigemina,* the onset of fever in acute infection is coincident with the presence of intraerythrocytic parasites, easily microscopically detectable at very low levels due to characteristic parasite morphology. In contrast, *B. bovis* may cause clinical disease with severe neurological signs followed by death with few detectable parasitized erythrocytes in peripheral blood. The sequestration of *B. bovis* parasitized erythrocytes in cerebral capillaries is readily identifiable in histological sections but not currently easily diagnosed *in vivo.* Onset of clinical anaplasmosis is associated with relatively high *A. marginale* rickettsaemias, greatly exceeding the minimum microscopically detectable level of approximately 0.05–0.1% infected erythrocytes. The simplicity of microscopic examination of stained blood smears and the utility for diagnosis of

multiple haemoparasites has ensured their frequent use in most of the world. In the United States, *A. marginale* remains the only significant haemoparasitic disease of cattle, consequently microscopic examination of blood smears has become an infrequently utilized technique with the concomitant loss of expertize in staining and examination. An antigen capture enzyme-linked immunosorbent assay (ELISA) has been recently developed (Trueblood *et al.,* 1991) that detects *A. marginale* in blood from acutely infected cattle. This rapid assay detects infection prior to the onset of severe anaemia, allowing early tetracycline therapy required for effective treatment of acute anaplasmosis. Significantly, the assay does not detect cattle with <2% rickettsaemia ensuring that only acutely infected cattle likely to require antibiotic treatment are identified (Trueblood *et al.,* 1991). Reactivity has been shown with all US strains of *A. marginale* tested to date. Whether this test for acute infection and for postmortem detection (using heart blood) will find common use among veterinarians and diagnostic laboratories remains to be seen. This type of antigen detection format may be useful for diagnosis of low level *B. bovis* parasitemia in cerebral babesiosis.

Chronic infection in cattle. Direct detection of persistently infected carrier cattle has been limited to either inoculation of suspect blood into susceptible (usually splenectomized) calves and test feeding of ticks with subsequent microscopic examination or tick transmission to susceptible cattle. The inability to examine large numbers of carriers and the lack of quantitation has severely limited the number of studies that can be done on the epidemiological significance of carrier cattle. The recent advent of nucleic acid probes for *A. marginale, B. bigemina* and *B. bovis* has provided the most sensitive and specific assays available for quantitation of infection. The published sensitivity and specificity for available probes are listed in Table 1. Use of nucleic acid probes has allowed improved definition of persistent infection in carriers. For example, probing of blood from chronic (>3 year) carrier cattle revealed cyclic variation in the *A. marginale* rickettsaemia level (Eriks *et al.,* 1989; Kieser *et al.,* 1990). These results indicated that the rickettsaemia in individual chronic carriers varies temporally from <0.000025% to >0.0025% and that direct detection of all carriers at a given time will require an assay sensitivity of <0.000025%. Significantly, these observations have spawned several new areas of investigation: i) influence of cyclic rickettsaemia on tick transmission; ii) mechanism of cyclic rickettsaemia with a focus on antigenic variation; and iii) use of polymerase chain reaction (PCR) to enhance sensitivity to allow uniform detection of all carriers. Optimization of sensitivity by PCR has become a routine technique and can be expected to be used with currently known or newly identified DNA sequences within the near future.

Infection of arthropods. Anaplasma marginale, B. bigemina and *B. bovis* infections of arthropod vectors have been detected using microscopic examination of haemolymph and tissue sections combined with transmission studies. More recently, nucleic acid probes have been used to detect and quantitate haemoparasites in vector ticks. Specifically, DNA probes have been used to identify *A. marginale* in *Dermacentor andersoni, D. variabilis* and *D. occidentalis* (Goff *et al.,* 1988) and *B. bigemina* in *Boophilus microplus* (Hodgson *et al.,* 1989, 1992). The sensitive detection of infection (as compared to microscopic examination), ability to quantitate levels of infection and the ability to assay large numbers of ticks provide new opportunities for epidemiological research. In addition DNA probes allow specific identification of small numbers of organisms in specific tick tissues (Stich *et al.,* 1990). Current

TABLE 1. Current nucleic acid probes for *Anaplasma* and *Babesia.*

Organism	Sensitivity DNA	Parasitemia	Specificity	Reference
A. marginale	0.5 pg	0.00025%	*A. marginale*	Barbet *et al.,* 1987; Eriks *et al.,* 1989; Goff *et al.,* 1988; Kieser *et al.,* 1990
			A. centrale	
A. marginale	100 pg	<0.001%	*A. marginale*	Aboyte-Torres and Buening, 1990; Aboyte-Torres *et al.,* 1989
			A. centrale?	
A. centrale	0.3 ng	ND	*A. centrale*	Visser and Ambrosio 1987, 1989
	0.6 ng	ND	*A. marginale*	
A. centrale	64 ng	ND	*A. centrale*	Visser and Ambrosio 1987, 1989
	ND	ND	*A. marginale*	
A. centrale	1.25 pg	ND	*A. centrale*	Visser and Ambrosio 1987, 1989
A. ovis	10 ng	0.0035%	*A. ovis*	Shompole *et al.,* 1989
	100 ng	ND [*]	*A. marginale*	
B. bigemina	10 pg	ND	*B. bigemina*	Buening *et al.,* 1990
B. bovis	100 pg	ND	*B. bovis*	McLaughlin *et al.,* 1986
	10 ng	ND	*B. bigemina*	
B. bovis	100 pg	ND	*B. bovis*	Jasmer *et al.,* 1990
	1 ng [†]	ND	*B. bigemina*	
B. bovis	100 pg [†]	ND [‡]	*B. bovis*	Jasmer *et al.,* 1990
B. bovis	12 pg	ND [‡]	*B. bovis*	Holman *et al.,* 1989

ND = Not determined.
[*]Detected <150 parasitized erythrocytes/μl.
[†]Sensitiv ity was reduced to 1ng for DNA from the Australia-L strain.
[‡]Positive detection of blood with approximately 1 parasitized erythrocyte/10 1000×microscope fields.

research being done based on existing probes includes: i) characterization of development of infective stages in the salivary gland (Blouin *et al.,* 1990); ii) influence of host parasitaemia level on tick infection and transmission; and ii) identification of small numbers of organisms following purification of infective stages from salivary glands (Blouin *et al.,* 1990; Hodgson *et al.,* 1989).

Fluorescein-labelled antibodies have been used to definitively identify organisms in tick tissue, however, to date, little research has been published on stage specific haemoparasite antigens. Identification of haemoparasite molecules required for invasion of host erythrocytes (Blouin *et al.,* 1990) would be of significant interest for both pathogenesis studies and vaccine development. The ability to purify and quantitate infective stage organisms (Hodgson *et al.,* 1992) should lead to new research in this area.

SEROLOGICAL DETECTION

The currently utilized serological assays for detection of *A. marginale-, B. bigemina-* or *B. bovis*-infected cattle have been reviewed previously (Kieser *et al.,* 1990). In

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summary, these assays are constrained by an unacceptably high number of false positives particularly in regions with multiple haemoparasitic infections and false negatives particularly in carriers. Current research is aimed at improvement of these assays by selection of species-specific, strain-common antigens which can then be produced by recombinant DNA expression. The principal advantage to recombinant DNA expression or use of synthetic antigens is that batch-batch variation in antigen production can be avoided. For each haemoparasite, polypeptides have been identified that meet the following criteria: highly antigenic following natural infection including long-term persistent infection in carriers; haemoparasite species specific; and conserved among different strains and isolates of the haemoparasite species (Table 2). Serum from long-term *A. marginale* carrier cattle consistently had high titres ($>10^4$) of antibody to the 86 kDa MSP-3 polypeptide (McGuire *et al.,* 1991; Palmer *et al.,* 1986). MSP-3 has conserved epitopes among strains isolated from widely geographically separated regions and shown to antigenically vary in other polypeptides (McGuire *et al.,* 1991; Palmer *et al.,* 1986). Serological detection of *A. marginale* carriers avoids the problem of cyclic rickettsaemia due to the constancy of antibody titre (McGuire *et al.,* 1991). Recombinant DNA clones expressing MSP-3 have been identified and sequenced. Strategies to obtain diagnostic antigen free of cross-reactive bacterial host antigens include synthetic peptides based on the translated sequence, recombinant expression as an inducible fusion protein with subsequent affinity purification and cleavage of the fusion protein and recombinant baculovirus expression in insect cell culture. Ideally cattle immunized with subunit antigens should not react with the diagnostic antigen. This would allow discrimination between immunized cattle and infected cattle, a significant advantage over current killed organism vaccines and serologic assays. Cattle immunized with *A. marginale* MSP-1 are unreactive with MSP-3 (unpublished data). In theory, challenged but protected cattle would remain seronegative or be only transiently seropositive to the diagnostic antigen. This hypothesis has not been tested due to the failure of subunit vaccines to provide consistent protection against infection upon haemoparasite challenge. The *B. bovis* 11C5 reactive polypeptide has been tested as a recombinant expressed polypeptide fragment (approximately 40 kDa) in an ELISA format and shown to be specific and sensitive (Bose *et al.,* 1990). Although promising, the use of this same antigen for subunit immunization may constrain its use as a diagnostic antigen if the same epitopes are included in both antigens. Additional *B. bovis* and *B. bigemina* polypeptides meeting criteria for application as diagnostic antigens have been identified but have not yet been extensively tested (see Table 2).

CONCLUSIONS

The availability of new reagents for identification of *Anaplasma* and *Babesia* provides new research opportunities, especially in epidemiology. The ability to identify and quantitate infection in carriers and arthropod vectors will allow determination of the variables, including acaricide use and vaccination, that affect development of individual animal and herd immunity. An improved understanding of herd immunity should lead to improved control over anaplasmosis and babesiosis in enzootic areas throughout the world.

TABLE 2. *Anaplasma* and *Babesia* polypeptides for diagnostic antigens.

Organism	Polypeptide	Specificity	Strain conservation	Reference
A. marginale	MSP-3	*A. marginale* *A. centrale*?	All tested (US, Zimbabwe)	McGuire *et al.,* 1991; Palmer *et al.,* 1986; Shkap *et al.,* 1991; Tebele *et al.,* 1991
B. bigemina	p58	*B. bigemina*	All tested (US, Kenya)	McElwain *et al.,* 1987, 1988
B. bigemina	p72	*B. bigemina*	All tested (US, Kenya)	McElwain *et al.,* 1987, 1988
B. bovis	ca. 40 kDa[*]	*B. bovis*	Australia (limited testing)	Bose *et al.,* 1990
B. bovis	Bv42	*B. bovis*	Strain (or regional strain) specific	Goff *et al.,* 1988, 1989; Hines *et al.,* 1989; McElwain *et al.,* 1988; Palmer *et al.,* 1991
B. bovis	Bv120	*B. bovis*	Limited testing	Goff *et al.,* 1988; Hines *et al.,* 1989; McElwain *et al.,* 1988

[*]Recombinant reactive with MAb 11C5 expressed in pGEX-1

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Serodiagnosis of *Cowdria ruminantium:* current status

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SUMMARY

Serological assays for detecting antibodies to *Cowdria ruminantium* were, until recently, of limited value because of the lack of sufficient quantities of good diagnostic antigen. The breakthrough in culturing large numbers of the organism in endothelial cell culture has drastically changed this situation. So far, two types of serological assays have been developed using *Cowdria* antigens harvested from these cell cultures. The indirect fluorescent antibody (IFA) test based on fluorescing extracellular elementary bodies of *Cowdria* which is being used in several laboratories. A competitive enzyme-linked immunosorbent assay has been developed more recently based on recognition by monoclonal antibodies of an immunodominant 32-kilodalton *Cowdria* protein from endothelial cell culture extracts. By using these serological assays a more accurate assessment of the prevalence and present distribution of heartwater in Sub-Saharan Africa and the Caribbean can be obtained.

INTRODUCTION

Heartwater, caused by the tick-borne rickettsia *Cowdria ruminantium,* occurs in domestic and wild ruminants in sub-Saharan Africa (Uilenberg, 1983). The disease is undoubtedly the most important tick-borne disease of small ruminants in Africa. Indigenous cattle populations in endemic areas appear to have a high degree of innate resistance to the disease, but for imported breeds including Zebu, heartwater represents the major tick-borne disease problem outside East Coast fever areas (Provost and Bezuidenhout, 1987). The existence of heartwater on the Caribbean islands of Guadeloupe, Marie-Galante and Antigua and the presence of *Amblyomma variegatum* on most of the islands in this region constitute a serious threat to livestock on the American mainland (Barré *et al.,* 1987).

For an accurate assessment of the present distribution of the disease in Africa and in the Caribbean, a reliable serodiagnostic assay is of prime importance. Serological assays for detecting antibodies to *C. ruminantium* were, until recently, of limited

value because of the lack of sufficient quantities of an effective antigen. Several tests have been developed based on antigen preparations obtained from infected host tissues. *Cowdria* antigens have also been produced using short-lived, granulocytic neutrophil cell cultures and most recently in endothelial cell cultures.

Host tissues as a source of *Cowdria* antigens

The first serological test for heartwater was a capillary flocculation test developed by Ilemobade and Blotkamp (1976). This test used an antigen extracted by acetone from brain material of infected goats or cattle. Antibodies were first detected one to two weeks after antibiotic treatment and remained at detectable levels for periods varying between one and four weeks. The short period of antibody detection limited the practical value of the test.

Complement fixation tests have been developed by du Plessis (1982) and Musisi and Hussein (1985). The latter authors used sucrose-acetone extraction of infected calf brain and blood and detected low titres of complement fixing antibodies in experimentally infected animals. However, these tests have not shown the required sensitivity and specificity.

Indirect fluorescent antibody test

The first IFA test was developed by du Plessis (1981) using infected peritoneal mouse macrophages as the source of *Cowdria* antigen. This test is based on the Kümm strain which is highly pathogenic for mice (du Plessis and Kümm, 1971). The test has shown its usefulness in serological monitoring of experimental animals in South Africa (du Plessis and Malan, 1987). For instance, the persistence of antibodies for three to seven months, with peak titres of 1:5,120, could be demonstrated in cattle following infection with the Ball 3 strain. Further, colostrum-derived antibodies to *Cowdria* were detected in sera from calves up to four weeks old. In lambs maternal antibodies were detected between 8 and 12 weeks after colostrum intake (du Plessis, 1984). In mice peak titres of 1:10,240 were detected and low titres persisted for 18 months. In sheep titres peaked at 1:20,480 and remained detectable for as long as 39 months, although at titres as low as 1:80. The persistence of antibody in these experimental animals for such long periods without reinfection suggests a long-term carrier state, assuming that no reinfection occurred. The test has not been widely used outside South Africa, except for Guadeloupe (Camus, 1987), mainly due to the difficulty in producing sufficient numbers of infected macrophages.

Enzyme-linked immunosorbent assays

Enzyme-linked immunosorbent assays (ELISA) using *Cowdria* antigens isolated from sheep brain or *A. hebraeum* ticks by wheat germ lectin affinity chromatography (Viljoen *et al.,* 1985; Neitz *et al.,* 1986b, 1986c) or Percoll density gradient centrifugation (Neitz *et al.,* 1986a) have also been reported. Viljoen *et al.* (1987) have

reviewed the theoretical aspects of the ELISA and its use in the detection of *C. ruminantium* antigens and antibodies in various heartwater-infected hosts. The main obstacles to large-scale use of these assays have been the purification of sufficient amounts of antigen from infected sheep or tick tissues.

Neutrophil cultures as a source of *Cowdria* antigens

Logan (1987) described an IFA test based on the discovery that *Cowdria* developed in neutrophilic granulocyte cultures (Logan *et al.,* 1987). A slightly modified test using these cultures as antigen was adopted in Utrecht, where it was shown to be suitable for several different *Cowdria* isolates (Jongejan *et al.,* 1989). However, not all isolates of *Cowdria* developed equally well in neutrophil cultures and some failed to do so. The *Cowdria*-infected neutrophils ranged from 1 to 30% and were highest after 24 to 72 hours. Thereafter the cultures degenerated quickly. Infected neutrophil cultures were used as antigen in a similar IFA test as described earlier by workers in Utrecht (Jongejan *et al.,* 1989) but the test differed to some extent from the original methods described by Logan (1987). Jongejan *et al.* (1989) found that it was important to reduce non-specific fluorescence, especially when using goat antisera on caprine neutrophil cultures, by pre-incubation with acid glycin buffer as described for the serodiagnosis of anaplasmosis (Montenegro-James *et al.,* 1985). Counter-staining with Evans Blue further reduced the non-specific fluorescence. Serological cross-reactions between five stocks of *Cowdria* were determined using goat antisera obtained six weeks after infection. All animals had been treated and given a homologous challenge two weeks before the sera were tested. In general, antibody titres detected using heterologous antigens in the test were much lower than on homologous antigens, demonstrating the presence of serotypes within the genus *Cowdria.* The same isolates were also different from each other with respect to their cross-immunity status in goats (Jongejan *et al.,* 1988, 1991c). However, it was not determined whether immunologically identical stocks of *Cowdria* belong consistently to the same serotype. The existence of serotypes within the genus *Cowdria* has reduced the usefulness of this IFA test based on infected neutrophils. For large-scale production of *Cowdria* antigens, infected endothelial cell cultures have been shown to be far superior (Bezuidenhout, 1987).

Infected endothelial cell cultures as a source of *Cowdria* antigen

The successful long-term *in vitro* propagation of *Cowdria,* first achieved in South Africa (Bezuidenhout *et al.,* 1985; Bezuidenhout, 1987), has been an important breakthrough. In Utrecht we have modified these original methods by using non-irradiated bovine endothelial cells isolated from umbilical cord arteries (Van de Wiel *et al.,* 1989). The details of isolation, maintenance of endothelial cell cultures and the behaviour of *Cowdria* within these cultures have been described in detail elsewhere (Jongejan, 1991). Researchers in Zimbabwe have reported several other improvements in the culture conditions for *Cowdria* (Byron and Yunker, 1990; Byron *et al.,* 1991). An IFA test based on fluorescing extracellular elementary bodies of *Cowdria*

has been developed using *Cowdria* antigens harvested from endothelial cell cultures (Yunker *et al.,* 1988; Martinez *et al.,* 1990). Both studies showed that the extracellular elementary bodies of *Cowdria* fluoresced brightly, whereas intracellular colonies of reticulate bodies fluoresced only faintly. Therefore, to determine the titre, the fluorescence emitted by the elementary bodies is recorded rather than searching for intracellular fluorescing bodies. This means that culture supernatant containing a large number of elementary bodies can be centrifuged at 10,000 g and the pellet spotted onto glass slides without any contaminating host cells. A large number of antigen slides can thus be prepared from one infected endothelial cell culture.

Comparison of IFA tests

Martinez and his colleagues (1990) have compared three antigens for the serodiagnosis of heartwater by IFA. Antigens were obtained from infected bovine endothelial cells (E5 cell line), peritoneal macrophages infected with the Kümm isolate, and primary cultures of infected caprine macrophages. The use of endothelial cell cultures as a source of *Cowdria* antigen proved superior in all respects. The antigen could be produced in large quantities at low cost, in contrast to the other two types of antigen described. The fluorescence was easy and quick to assess compared to the laborious reading of neutrophil or macrophage antigens, which often contain only a low percentage of small rickettsial colonies.

The demonstration of serotypes of *C. ruminantium* using the IFA test on infected neutrophils stressed the need for a *Cowdria*-specific test detecting all serotypes (Jongejan *et al.,* 1988). The problem of serotypes has also been addressed by Martinez *et al.* (1990) while evaluating the IFA based on endothelial cell culture antigen. The Welgevonden isolate from South Africa, an isolate from Senegal and the Gardel isolate from Guadeloupe showed lower heterologous titres compared to homologous titres. Further, a number of Zimbabwean isolates have also been compared using the IFA test and it was found that all the isolates were essentially of the same serotype (Yunker, 1990).

Monoclonal antibody-mediated competitive ELISA

Unpurified *Cowdria* antigens harvested from infected endothelial cultures did not give satisfactory results in the indirect ELISA due to the high background obtained with pre-immune sera (M.J.C. Thielemans and F. Jongejan, unpublished observations). These non-specific reactions associated with crude antigen could be avoided by using the specificity of monoclonal antibodies (Hewitt *et al.,* 1982). For instance, a competitive ELISA mediated by a monoclonal antibody has also been used for the serodiagnosis of *Ehrlichia risticii,* the causal agent of equine monocytic ehrlichiosis (Shankarappa *et al.,* 1989).

It was demonstrated recently that *Cowdria* contains an immunodominant and antigenically conserved protein of 32,000 daltons (Jongejan and Thielemans, 1989). Monoclonal antibodies (MAb) directed against this dominant protein, designated Cr32, were subsequently raised. These MAb were used to determine the localization

of the Cr32 protein and to develop a competitive ELISA for the detection of circulating antibodies. A panel of five MAb were raised against *Cowdria.* Four MAb were directed against epitopes on a 32-kilodalton *Cowdria* protein as demonstrated by Western blotting; two (4F10B4 and 1E3H10) were of the IgG3 isotype and the two others (3D8H1 and 1E5H8) of the IgG2b isotype. A fifth MAb (4B2F3H12) of the IgM isotype recognized a 40-kDa *Cowdria* protein. All four anti-Cr32 MAb were reactive in an IFA test based on extracellular elementary bodies. The anti-40 kDa MAb was negative in this test. MAb 4F10B4 showed the strongest signal in Western blots using three different stocks of *Cowdria.*

To determine whether the Cr32 protein has surface exposed antigenic determinants, immuno-gold labelling of *Cowdria in vitro* using 4F10B4 was carried out according to methods originally designed by Faulk and Taylor (1971) and adapted for *in vitro* labelling of *Chlamydia* elementary bodies by Kuo and Chi (1987). A suspension of *Cowdria* elementary bodies was allowed to react with MAb 4F10B4 and subsequently labelled with colloidal gold conjugated to anti-mouse IgG. The material was processed for transmission electron microscopy (Ito and Rikihisa, 1981). It was shown that Cr32 has surface-exposed antigenic determinants (Jongejan *et al.,* 1991b) expected on the basis of its immunodominant recognition.

Using MAb 4F10B4, a competitive ELISA was developed which detected *Cowdria*-specific antibodies in goat, sheep and cattle sera (Jongejan *et al.,* 1991b). In this test antibodies in animal sera compete with MAb for binding sites on the Cr32 protein present in a crude sonicated *Cowdria* extract obtained from infected endothelial cell cultures. For instance, competition between anti-Cr32 antibodies in bovine sera and MAb results in a lower extinction since the rabbit-anti-mouse second antibody does not recognize bovine antibodies.

The competitive ELISA detected antibodies in 55 out of 70 (79%) goats experimentally infected with one of eight different *Cowdria* stocks. Fourteen out of the 15 sera which were negative in the competitive ELISA were also negative in the IFA test. Nevertheless, all 15 sera recognized some epitopes of the Cr32 *Cowdria* protein as judged from their reaction with this protein in Western blots of infected endothelial cell culture extract. Overall, there was 89% agreement between the competitive ELISA and the IFA test considering all 70 goats. Moreover, antibodies were detected in 13 out of 13 sheep infected with one of four different stocks of *Cowdria,* and in sera from calves experimentally infected with six different strains of heartwater. So far experimental animals immunized against 12 different isolates of *Cowdria* were recognized in the competitive ELISA (five isolates from South Africa; Ball 3, Kümm, Kwanyanga, Zeerust and Welgevonden, Gardel from Guadeloupe, Lutale from Zambia, Kiswani from Kenya, Ifé from Nigeria, Umm Banein from Sudan, Umpala from Mozambique and Cristal Springs from Zimbabwe).

The results show that in the competitive ELISA serotype-specificity does not play a role. This may have been expected since Cr32 is a conserved protein present in all isolates of *Cowdria* tested so far. Comparison of the competitive ELISA with IFA test results for goat sera showed 89% agreement. The competitive ELISA detected antibodies in seven goat sera, which were negative in the IFA test, indicating that the competitive ELISA is more sensitive. Both tests failed to detect antibodies in 14 sera which were obtained from goats immunized against different *Cowdria* stocks. However, when these sera were tested in Western blots they all appeared to recognize the

CR32 protein. A possible explanation is that polyclonal goat antisera recognize a different epitope on the CR32 molecule than the monoclonal antibody. The Western blot appears to be a more sensitive assay for the detection of antibodies against the *Cowdria*-specific protein. However, since Western blotting is not very practical for large-scale serological testing, the technique is used as an important additional method of evaluating previous exposure to *Cowdria* infections.

The advantage of the competitive ELISA is that sera from many animal species can be tested, but not the mouse. Our preliminary results in Zimbabwe and Mozambique with sera from wild animals indicate that the competition ELISA can also be used to determine the involvement of wildlife in the epidemiology of heartwater.

Cross-reactions

Ehrlichia

Another important characteristic of an effective serological test is the absence of cross-reactions with other organisms which may lead to false positive results. It was shown by Logan *et al.* (1986) that the specificity of the IFA test based on infected neutrophil cultures was limited due to cross-reactions between *Cowdria* and *Ehrlichia* (Camus, 1987; Jongejan *et al.,* 1989). A strong cross-reaction was found between *Cowdria* antigens and *E. equi* antisera and to a lesser extent with *E. canis* (Logan *et al.,* 1986). Moreover, two-way cross-reactions between *Cowdria* and *E. phagocytophila* with titres between 1:80 and 1:160 were found when these organisms were compared in neutrophil cultures by workers in Utrecht (Jongejan *et al.,* 1989).

Similar cross-reactions have been reported using the IFA based on infected macrophages. Unilateral cross-reactions were found with *E. bovis, E. phagocytophila, E. ovina* (one only out of two sera tested) and antisera to an *Ehrlichia* sp. associated with Jembrana disease in Indonesia (Camus, 1987; du Plessis *et al.,* 1987). Cross-reactivity between *Cowdria* and *E. phagocytophila* is not important in the epidemiology of heartwater since the infections occur on different continents. Moreover, this cross-reactivity is not linked to cross-immunity between the organisms (Jongejan and Wassink, 1991). Cross-reactions with other *Ehrlichia* species, such as *E. bovis* or *E. ondiri* could interfere with the interpretation of sero-epidemiological surveys of heartwater (du Plessis *et al.,* 1987). Although serological cross-reactions limit the value of the IFA test based on macrophages in the epidemiology of heartwater, the test remains a valuable tool to monitor infections in experimental animals. It remains to be shown if, and to what extent, cross-reaction with *Ehrlichia* spp. may play a role in the IFA test based on elementary bodies harvested from endothelial cell cultures.

Ehrlichia phagocytophila positive sera, shown previously to cross-react in the IFA test with *Cowdria*-infected neutrophils, did not cross-react in the competitive ELISA. Also, in Western blots no epitopes on the Cr32 molecule are recognized by *E. phagocytophila* antibodies. Although it seems unlikely that any other ehrlichial agents will recognize epitopes on this *Cowdria*-specific protein, further studies on this aspect of the competitive ELISA are required, especially with *E. bovis* and *E. ondiri* which occur sympatrically with *C. ruminantium.*

Chlamydia

The relationship between *C. ruminantium* and *Chlamydia trachomatis* has been studied by immunofluorescence. A monoclonal antibody directed against the MOMP of *Chlamydia trachomatis* recognized rickettsial colonies of *C. ruminantium* in infected goat brain (Jongejan *et al.,* 1991a). No specific fluorescence was observed in non-infected brain. Also, polyclonal *Cowdria* antiserum did not recognize *Chlamydia trachomatis* in cultured HeLa cells, whereas *Chlamydia*-specific polyclonal rabbit antiserum did. Western blotting analysis using the *Chlamydia* monoclonal antibody on nitrocellulose blots containing *Cowdria* proteins from endothelial cell cultures, did not identify proteins with common antigenic determinants. The loss of reactivity of the MAb in Western blots may have been due to protein denaturation during SDS/PAGE. It was concluded that in addition to the existing similarities in ultrastructure and developmental cycle, *Cowdria* and *Chlamydia* are to a certain extent antigenically related (Jongejan *et al.,* 1991). However, further assessment of the relationship between these organisms based on molecular phylogenetic analysis using 16S ribosomal DNA sequences has revealed that *Cowdria* is closest related to several species of *Erhlichia* (Van Vliet *et al.,* 1992).

Bovine abortion rickettsia (BAR)

An obligate intracellular rickettsial organism was recently isolated from tissues of an aborted bovine foetus in the USA (Dilbeck *et al.,* 1990; Kocan *et al.,* 1990). A high cross-reactivity was found between this newly isolated rickettsia and *Cowdria.* In collaboration with Washington State University, Pullman, and the SADCC/ Heartwater Research Project carried out by the University of Florida in Zimbabwe, the antigenic relationship between this BAR agent and *Cowdria* was re-examined using IFA, competitive ELISA and Western blotting. The results obtained did not confirm the previously claimed cross-reactivity between this new agent and *Cowdria.*

Evaluation of competitive ELISA and the IFA test

Mozambique

Heartwater appears to be a major disease problem in improved cattle and goats south of the River Save in Mozambique; it is much less important in the south of the country. The vector in the north is *A. variegatum* and in the south is *A. hebraeum.* The IFA test using supernatant from infected endothelial cell culture (from Utrecht) as antigen was used to test 388 caprine sera and 374 bovine sera collected in six provinces (Faculdade de Veterinária, Maputo). It was shown that north of the River Save, where only *A. variegatum* is present, the percentages of positive sera were low (10% for goats and 20% for cattle), whereas south of the River Save, where *A. hebraeum* is abundant, percentages were much higher (63.5% for goats and 59.5% for cattle). Thus

the differences in the disease picture in regions with *A. hebraeum* or *A. variegatum* could be confirmed serologically (Asselbergs *et al.,* in press).

Malawi

Cowdria antigen produced at Utrecht in endothelial cell cultures was evaluated in Malawi (FAO Project GCP/RAF/259/DEN in Lilongwe). It was found that the IFA test based on elementary bodies of *Cowdria* was a sensitive indicator of the establishment of infection after immunization of calves with frozen blood vaccine. Significant differences in infectivity were demonstrated between four batches of vaccine. Seroconversion occurred in between 72 and 100 percent of animals after inoculation of a reference vaccine of proven viability (J.A. Lawrence, A.P. Whiteland, J. Malika, P. Kafuwa and F. Jongejan, unpublished data).

Zimbabwe

The competitive ELISA was introduced in Zimbabwe at the Paraclinical Department of the Faculty of Veterinary Science. Serum samples from 36 free ranging black rhinoceroses *(Diceros bicornis)* from the Zambezi Valley in Zimbabwe (obtained from the Department of National Parks and Wildlife Management) were tested for exposure to *Cowdria* using the competitive ELISA. Nineteen (53%) of the animals were positive, indicating exposure in the natural habitat where there is no contact with domestic cattle. The findings signify a possible role for the black rhinoceros in the epidemiology of the disease (N.D. Kock, F. Jongejan, M.D. Kock and R.A. Kock, unpublished results).

Guadeloupe

The competitive ELISA has been evaluated in the Caribbean region on Guadeloupe where cattle and goat sera were tested for antibodies directed against the CR32 protein of *Cowdria.* All sera obtained from livestock on islands free from *A. variegatum* were negative. However, low numbers of sera from cattle on islands other than those where heartwater has been demonstrated, were found positive by competitive ELISA and the IFA test and these results have been confirmed using Western blotting analysis at Utrecht. A serological survey of heartwater in the Caribbean, carried out jointly by workers in Guadeloupe and Utrecht, will be described elsewhere (Muller-Kobold *et al.,* 1992).

ACKNOWLEDGEMENTS

from the laboratory in Utrecht and elsewhere for their contributions to the heartwater research.

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DNA probes for *Cowdria ruminantium*

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INTRODUCTION

Heartwater is an economically important disease of livestock caused by *Cowdria ruminantium* and transmitted by ticks of the genus *Amblyomma.* Current methods for the diagnosis of heartwater in ticks and animals are either slow or are considered to have inadequate specificity and sensitivity. Demonstration of *C. ruminantium* in ticks and in live animals is difficult and often requires sub-inoculation into susceptible animals. Definitive diagnosis of heartwater in animals is made by identifying the organisms in endothelial cells in Giemsa-stained brain smears (Purchase, 1945) obtained by biopsy or at postmortem. In infected ticks, colonies are found by thick or thin sections of midgut by light and electron microscopy (Bezuidenhout, 1984; Kocan and Bezuidenhout, 1987; Yunker *et al.,* 1987). RNA and DNA probes have been developed to detect rickettsia and other organisms in animal hosts and arthropod vectors by nucleic acid hybridization (Barker *et al.,* 1986; Goff *et al.,* 1988; McLaughlin *et al.,* 1987; Shompole *et al.,* 1989) and have been acclaimed to have high specificity and sensitivity. Bearing this in mind, we have identified a DNA probe for the diagnosis of heartwater.

IDENTIFICATION OF A DNA PROBE
SPECIFIC FOR *COWDRIA RUMINANTIUM*

A DNA probe has been identified for the diagnosis of heartwater in ticks and animals. The probe has been derived from the DNA of the Crystal Springs heartwater isolate of Zimbabwe (Waghela *et al.,* 1991). This probe has been isolated from a genomic DNA library prepared by cloning of *Sau*3A partially digested DNA into the BamH1 site of pUC 19 plasmid vector. Recombinant plasmids from transformed *Escherichia coli* were identified by alkaline lysis, followed by digestion with EcoR1 and electrophoresis on 0.6% agarose gels. Those plasmids with inserts were digested with Kpn1 and Xba1, Southern blotted and probed with infected and uninfected endothelial cell DNA. A plasmid carrying a 1306 base pair *C. ruminantium* DNA fragment was identified by its hybridization to infected but not uninfected endothelial cell DNA and was called pCS20 (Waghela *et al.,* 1991).

CHARACTERIZATION OF pCS20 PROBE

The specificity and sensitivity of pCS20 for Crystal Springs DNA was determined (Waghela *et al.,* 1991). The ^{32}P-labelled pCS20 probe had high specificity for *C. ruminantium* DNA as it did not hybridize in a dot blot assay to 400 ng of *Anaplasma marginale, Trypanosoma brucei, Babesia bigemina, B. bovis, Ehrlichia risticii,* a new bovine *Chlamydia*-like organism, *E. coli, Campylobacter jejuni, C. pneumoniae, C. fetus, Staphylococcus aureus, Pseudomonas aeroginosa, Streptococcus faecalis, Clostridium perfringens, Actinobaccilus pyogenes, Listeria monocytogenes* and goat and bovine DNA (Waghela *et al.,* 1991). It was determined that the pCS20 probe had a sensitivity of detection of 1 ng of Crystal Springs genomic DNA which is known to be contaminated with bovine DNA, as the DNA was prepared from *in vitro* cultures of *C. ruminantium.* The specificity of the pCS20 probe was further characterized by Southern blotting of Crystal Springs DNA. In this assay it was evident that the pCS20 probe hybridized with four fragments of the Pst1 digested DNA of the isolate (Waghela *et al.,* 1991).

SEQUENCE ANALYSIS OF pCS20

The nucleotide sequence of both strands of pCS20 insert revealed a fragment length of 1306 base pairs containing two open reading frames of 513 and 459 nucleotides and contained approximately 70% A+T which caused numerous potential prokaryotic promoter consensus sequences (Waghela *et al.,* 1991).

CROSS REACTION OF pCS20 WITH OTHER HEARTWATER ISOLATES

The reaction of the pCS20 probe was determined against several other isolates of *Cowdria* from Zimbabwe, South Africa, Kenya and Nigeria. These analyses revealed that the probe hybridized to all heartwater isolates tested (Figure 1)
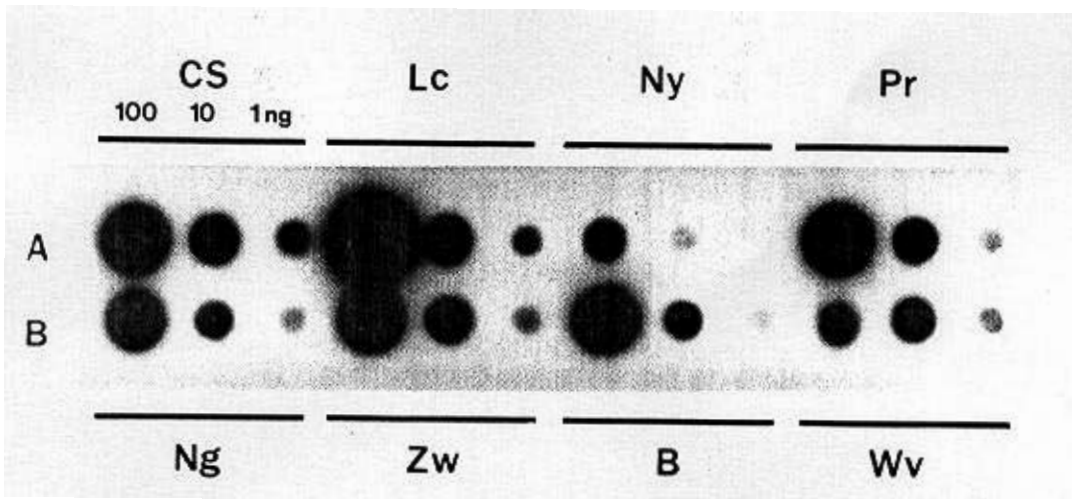
FIGURE 1. Hybridization of pCS20 DNA probe (^{32}P-labelled) with DNA from cultured elementary bodies of eight different isolates of *Cowdria ruminantium.* CS[*] = Crystal Springs; Lc[*] = Lemco T3; Ny[*] = Nyatsanga; Pr[*] = Palm River; Ng = Nigeria; Zw[*] = Zwimba; Ball 3 and Welgevondon (South Africa). (*Zimbabwe isolates)

regardless of their geographical origin, suggesting that it detects conserved DNA sequences in all the isolates and can be used to detect the parasite in different endemic regions of Africa.

DETECTION OF *COWDRIA RUMINANTIUM* IN *AMBLYOMMA VARIEGATUM*

The pCS20 probe has been shown to detect *C. ruminantium* organisms in *A. variegatum* ticks. The probe detected *C. ruminantium* DNA in 31 of 70 midgut DNA of adult ticks fed as nymphs on goats infected with the Kiswani isolate of Kenya, but not with ticks fed on uninfected goats (Waghela *et al.,* 1991). Ticks fed on infected goats which were negative with the probe were either uninfected or were infected at levels that were below the sensitivity of detection of the pCS20 probe.

DETECTION OF *COWDRIA RUMINANTIUM* IN INFECTED SHEEP

Five sheep inoculated with *C. ruminantium* Crystal Springs tissue culture organisms were sampled every other day until day 14 after infection. *Cowdria ruminantium* DNA was detectable in elementary body pellets from plasma of all infected sheep, five days after infection, by hybridization to the pCS20 probe. The maximum hybridization signal of the pCS20 probe with the pellet DNA from the plasma was detected on day 10 after infection in all but one animal, and coincided with the peak of the febrile reaction. The last animal died on day 9 after infection during the febrile reaction without showing a peak reaction with the pCS20 probe. No hybridization

signals were detected with plasma from five uninfected control sheep. The probe's sensitivity to detect *C. ruminantium* organisms in recovered or carrier animals is under investigation.

FURTHER DEVELOPMENTS OF THE pCS20 PROBE FOR THE DIAGNOSIS OF HEARTWATER

Synthetic oligonucleotides have been prepared from the sequence of the pCS20 probe in order to amplify, by the polymerase chain reaction (PCR), 181 bp and 279 bp products from the two open reading frames of the probe. An evaluation of the use of such PCR products for the detection of *C. ruminantium* will be presented. Such PCR-derived DNA products of pCS20 should reduce the hybridization time of the probe to test samples. Alternatively, PCR will be used to amplify the products described above from animal and tick DNA samples, which will be analysed by agarose gel electrophoresis and/or Southern blotting to confirm *Cowdria* infection. This method may be particularly useful for the detection of carrier animals which may have very low levels of intermittent parasitaemia. To allow routine use of a DNA-based diagnostic test for *Cowdria* in the field, a non-radioactive detection method using chemiluminescence is being developed. Initial results suggest that this method of detection has a similar sensitivity to the radiolabelled assay. Application of such a test would facilitate better understanding of the epidemiology of heartwater and hence allow the development of better control strategies for the disease.

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APPROACHES
TO NEW VACCINES
AND CONTROL MEASURES

Molecular biology of rickettsiae: gene organization and structure in *Anaplasma marginale* and *Cowdria ruminantium*

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INTRODCUTION

Important features of surface protein gene structure of *Anaplasma marginale* have been elucidated in the last ten years. The surface protein complex for major surface protein MSP1 of *A. marginale* has been shown to induce protection in cattle against homologous (Palmer *et al.,* 1986) and heterologous (Palmer *et al.,* 1989) challenge. Genes coding for two antigenically and structurally distinct polypeptides in this complex, MSP1a and MSP1b, have been cloned, sequenced and expressed (Barbet *et al.,* 1987; Barbet and Allred, 1991; Allred *et al.,* 1990). From these structures several conclusions can be drawn concerning surface protein gene organization. For *Cowdria ruminantium,* the availability of new culture methods (Bezuidenhout, 1987; Yunker *et al.,* 1988) suggests that similar advances can now be made. Research on *C. ruminantium* is at an earlier stage of development, particularly concerning the nature of protective immune mechanisms. However, genomic DNA expression libraries have been prepared from *C. ruminantium* and colonies expressing fragments of immunodominant proteins identified. This suggests that previous limitations imposed by lack of purified organisms or of cloned genes important in generation of immunity can be overcome.

MATERIALS AND METHODS

Cloning and expression of genes coding for polypeptide MSP1a (termed MSP1α genes). Genomic DNA was purified from the Florida isolate of *A. marginale* as previously

described (Barbet *et al.,* 1987). DNA libraries were prepared in the plasmid vectors pUC9 and pKK233-2. Recombinant *Escherichia coli* were screened for expression of MSP1a by reactivity with neutralizing monoclonal antibody Ana22B$_1$ which was detected by incubation with rabbit anti-mouse immunoglobulin and ^{125}I-protein A (Barbet *et al.,* 1987). Individual colonies were isolated that expressed as either fragments of MSP1a or the complete polypeptide. DNA was also prepared from the Virginia (VA), South Idaho (ID) and Washington-O (WA) isolates of *A. marginale.* Different cloning strategies were used to obtain the MSP1α gene from these isolates: ligation of KpnI (VA) or KpnI/ PstI (ID and WA) fragments into pGEM4 and screening of transformants by colony hybridization with probes made from fragments of the FL allele.

Cloning and expression of the gene coding for polypeptide MSP1b (termed MSP1β gene). DNA libraries were prepared in the plasmid vector pBR322 and screened for expression as above except that a rabbit antiserum against the MSP1 complex was used. Recombinant colonies were identified that expressed a protein of identical molecular weight and proteolytic cleavage pattern to MSP1b (Barbet *et al.,* 1987).

Sequencing of the cloned DNAs. Sequencing was performed on the double-stranded plasmid DNAs and subclones in pGEM3 and pGEM4 by the dideoxynucleotide method of Sanger *et al.* (1977). The t7 and SP6 promoter primers were used to sequence from pGEM into the insert, then new primers were made based on the newly acquired sequence data. The new primers were used to extend the region sequenced. By continuing this process of `primer-walking', both strands of the inserts were completely sequenced.

Synthetic peptide immunoblots. Nitrocellulose filters were soaked in a solution of 1 mg/ml bovine serum albumin (BSA) for 15 minutes and allowed to air dry. Peptides were then applied to the BSA coated nitrocellulose in a volume of 1 μl and the spots also allowed to dry. The nitrocellulose filters were wetted from behind by laying them for 15 minutes on a piece of 3MM paper that had been soaked in 2.5% glutaraldehyde. Filters were then washed × 3 rapidly in 0.01 M tris, pH 7.5, 0.17 M NaCl, 0.1 mM phenyl methyl sulfonyl fluoride and processed for reaction with 2 μg/ml monoclonal antibody, rabbit anti-mouse immunoglobulin, and ^{125}I-Protein A as described previously (Barbet *et al.,* 1987).

RESULTS AND DISCUSSION

Sequencing of DNA from recombinant bacteria expressing MSP1a. Subcloning of the *A. marginale* insert DNA in different orientations and plasmids, followed by immunoscreening, suggested that expression of recombinant MSP1a was occurring under control of an *A. marginale* promoter. Sequencing of the complete (Florida) DNA insert revealed a single long open reading frame potentially coding for a polypeptide of 767 amino acids (Allred *et al.,* 1990) and calculated molecular weight 77,454. A prominent feature of the sequence was the presence of eight tandemly repeated segments containing either 84 or 87 base pairs just 3' to the initiation codon (Allred *et al.,* 1990). This long open reading frame is the likely coding sequence because:

a) plasmid DNA deletion experiments showed that the presence of the tandem repeats was essential for reaction of recombinant bacteria with monoclonal antibody $22B_1$;

b) synthetic peptides specified by this open reading frame reacted with neutralizing monoclonal antibodies (see later);

c) the presumptive coding sequence was transcribed by *A. marginale* into RNA. The 5' end of the transcript was correctly spaced with regard to DNA sequences homologous to the –10 and –35 consensus promoter sequences of *E. coli;*

d) recombinant colonies expressing fragments of MSP1a contained the 5' region of this sequence including promoter, initiation codon and repeats, but were missing the 3' end; and

e) the N-terminal sequence of isolated native MSP1a agreed with that specified by the open reading frame.

The molecular weight of the complete polypeptide predicted from the sequence is less than its apparent molecular weight on SDS gels. This is a common finding with polypeptides containing tandem repeats (Kemp *et al.,* 1987) and agrees with our observation that a recombinant fragment of the MSP1a polypeptide containing only the repeat region also migrated anomalously.

Mapping of the epitope recognized by neutralizing monoclonal antibodies. A series of overlapping peptides were synthesized that were specified by different regions within the 87 base pair repeats of the Florida MSP1α gene. Each peptide was tested for reaction with monoclonal antibodies $22B_1$ and $15D_2$ in immunoblots and ELISA. Their reactivity in each assay and with both antibodies was comparable. The minimal epitope required for binding was the 6 amino acid sequence, QASTSS, which is present in seven of the peptide repeats. An alternative form of this sequence EASTSS (a conservative substitution of glutamic acid for glutamine in the N-terminal amino acid) exists in one repeat and also bound antibody. Shorter peptides missing either the N- or C-terminal amino acids of QASTSS and negative peptide controls did not bind. The 29 amino acid peptide representing one complete repeat, DSSSAGGQQQESSVSSQSD**QASTSS**QLGA, clearly bound more antibody than either of the 6 amino acid peptides. This suggests that although the epitope can be represented by QASTSS or EASTSS, the surrounding amino acids are important in maintaining an optimal conformation for binding.

Antisera which had been prepared previously in rabbits and cattle against non-recombinant MSP1 complex isolated from *A. marginale* infected erythrocytes (Palmer *et al.,* 1986) reacted well with the synthetic 29 amino acid peptide in the ELISA, demonstrating that this is an immunodominant part of the molecule. No reaction was obtained between these antisera and shorter peptides.

Comparison of the MSP1α gene in different Anaplasma marginale *isolates.* Restriction maps developed from chromosomal DNAs by Southern blot analysis revealed that the MSP1α gene was contained in a KpnI fragment that varied considerably in length (e.g. from 6.1 Kbp in the VA isolate to 6.6 Kbp in FL). This restriction fragment length polymorphism correlated with the variability in the apparent sizes of the MSP1a polypeptides from the different isolates (Figure 1). Because of the regions of the chromosome contained in the various plasmids and the fact that the plasmids each

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K                      H        B     SmP     S Hp         S    P              PSm  K
I-/ABBBBBB——-I———————I/—-I I———-I-I———-I—I— 1——-I-I-I FL
   K                   H        B     SmP     S Hp         S    P              PSm  K
  I-/AB——-I———————I/—-I I———-I-I———-I—I— 1——-I-I-I VA
   K                   H        B     SmP     S Hp         S    P              PSm  K
  I-/BBBC——-I———————I/—-II ———-I-I———-I—I— ——-I-I-I WA
   K                   B        H     SmP     S Hp         S    P              PSm  K
  I/DDDDDE———————————I/I 1II——-I-I———-I—I— @151——-I-I-I ID
^—————-^————-^—@151——-^————-^——-^ 51—-^————-^————— -^
0 Kbp  1.0     2.0     3.0     4.0     5.0     6.0    7.0
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FIGURE 1: Restriction map of the MSP1α gene and region 3' to the gene, from four allelic variants. The region of the chromosome containing the coding sequence of each MSP1α allele is indicated by bolding and delineated by slashes. The strings of A, B, C, D and E at the left end of each refer to the form and number of each repeat unit found in each allele. K= KpnI; H= HindIII; B= BamHI; Sm= SmaI; P= PstI; S= SstI; Hp= HpaI.

directed the synthesis of full-sized, immunoreactive polypeptides, we determined that the entire MSP1α gene was contained in the variable KpnI to PstI fragment and not in the constant surrounding regions.

The most unusual feature of the MSP1a polypeptide immediately follows the N-terminal domain: a region of tandemly repeated amino acids in which repeat units of 29 or 28 amino acids are repeated two (VA), four (WA), six (ID) or eight (FL) times. Five different repeat forms exist in the four isolates; of these, 25 of the amino acids are completely conserved in all forms. The variability in the numbers of repeats can completely account for the observed variations (Oberle *et al.,* 1988) in the size of this polypeptide.

Sequencing of DNA from recombinant bacteria expressing MSP1b. The cloned Florida MSP1β gene was mapped to a single long open reading frame coding for a protein of 756 amino acids and a calculated molecular weight of 80,350. As with the MSP1α gene, *A. marginale* RNA was sequenced to identify the transcription start site and the N-terminal sequence of native MSP1b was obtained to confirm location of the initiation codon. A typical *E. coli* –10 consensus sequence was observed in the expected location with respect to the transcription start. A domain containing four tandem repeats of a degenerate 7 amino acid sequence was observed. A second unusual feature was the presence of a run of four or five glutamine residues 6 amino acids in from the N- and C-termini (Barbet and Allred, 1991). There appeared to be three to four partially homologous copies of the MSP1β gene in genomic DNA, of which at least two occur in tandem.

Implications for vaccine development. Availability of complete gene sequences for MSP1a and b allows several possibilities for construction and testing of recombinant and synthetic products. Similar work on expression of recombinant immunodominant *C. ruminantium* proteins is in progress. These proteins and the cloned genes will be used in delineation of protective immune mechanisms and vaccine development.

ACKNOWLEDGEMENTS

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Methodological approaches to the study of resistance to cowdriosis and the possible role of cell-mediated immunity

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RESISTANCE TO COWDRIOSIS

Reports that populations rather than breeds of domestic animals could be resistant to cowdriosis were first mentioned by Uilenberg (1983). Although the observation that indigenous populations can survive in endemic areas while imported, exotic animals are fully susceptible to the disease is of importance, interpretations on the status of resistance can be biased due to the fact that very young animals are naturally resistant to cowdriosis. If not infected within a few weeks after birth, these animals become fully susceptible. Therefore, investigations of genetic resistance as opposed to immunity need to be performed in areas where the epidemiology of cowdriosis is well understood. Since cowdriosis was diagnosed in Guadeloupe (Perreau *et al.,* 1980), a complete epidemiological study has been performed on this and neighbouring islands. This has allowed the identification of areas free of ticks and therefore of the disease, areas in which the number of infected ticks has been assessed and livestock populations which have been in contact or not with the causal organism, *Cowdria ruminantium.* Furthermore, because importation and movement of livestock is well controlled and the *C. ruminantium* strain isolated in Guadeloupe is highly pathogenic, all conditions necessary for the study of genetic resistance to cowdriosis are fulfilled (Camus, 1989).

Currently, 95% of the livestock in Guadeloupe consists of Creole breeds which have been adapted to the environment. This population, mainly cattle and goats, was imported from West Africa in the 19th century. Importation from Africa stopped with the end of the slave trade. Goats first populated Basse Terre and Grande Terre, the two main islands of Guadeloupe, and were exported to the nearby island of Les Saintes, which is free of ticks.

Resistance of cattle to cowdriosis

In the absence of tick control, mortality due to cowdriosis is only 1% for the Creole breed while it increases to 10% for European breeds and 5% for cross-breds. Since only 1% of ticks are infected by *C. ruminantium,* it has been estimated that less than half of the cattle population is challenged each year. This observation implies that

few newborn animals are challenged during the period of natural age-related immunity, which lasts three to six weeks after birth. As a result, many animals are not naturally immune to the disease and differences in mortality between breeds are significant in terms of resistance to cowdriosis (Camus 1989). However, before stating that Creole cattle are resistant to cowdriosis, experimental infections need to be performed on animals born in tick-free areas.

Resistance of goats to cowdriosis

The most devastating effects of cowdriosis in Guadeloupe are in goats. In the absence of tick control, the mortality due to *C ruminantium* infection is 20% (Camus 1989). Therefore, goats are more susceptible to cowdriosis than cattle. For economical and practical reasons Creole goats were first used as experimental animals to study the transmission of the parasite. It was noticed that the susceptibility to cowdriosis varied from animal to animal within a herd and from herd to herd within the Creole population. In order to further investigate whether this variation in susceptibility or resistance was genetically controlled, population and family studies were performed. Three herds from three different locations were studied. Creole goats from Les Saintes have been evolving in a cowdriosis-free environment for the last 100 years while the population from the Duclos farm (Basse Terre) has been free from the disease for the last ten years. This population was derived from the Gardel farm (Grande Terre) which is an endemic area for cowdriosis. The numbers of animals subjected to experimental infection were 85, 90 and 36 from the Les Saintes, Duclos and Gardel populations, respectively. None of the animals were treated and the survival rates were 25, 54 and 78% respectively. A correction factor was introduced to the data obtained from the Gardel farm in order to account for a possible effect due to animals already immune. Also, a parallel experiment using the same *C. ruminantium* strain was carried out in Holland on 12 Dutch goats and none survived (Matheron *et al.,* 1987).

Of the 90 goats from the Duclos farm tested, 77 had identified parents and were the descendents of 19 sires. Seven sires had more than four offspring whose survival rate varied from 20 to 83%. The population comprised ten full-sib pairs of which nine reacted similarly, either both died or both survived. From these observations, it was concluded that, most likely, cowdriosis resistance was genetically controlled and a recessive sex-linked gene may have been involved. However, definite conclusions could not be drawn because of the small numbers studied (Matheron *et al.,* 1987).

METHODS AND STRATEGIES TO FURTHER INVESTIGATE RESISTANCE TO COWDRIOSIS

Establishment of Creole goat families

It is clear from the results described above that some Creole goats are resistant to cowdriosis. Techniques of artificial insemination and embryo splitting are well established and will be applied to obtain goat families with large numbers of full- and

half-sibs. These families will be valuable in the exploration of paternal effects on resistance and to study the heritability of characters conferring resistance to cowdriosis. Sperm from susceptible animals will be kept in order to establish susceptible families, and embryos from suspected susceptible females inseminated by susceptible males split. One half of the embryo will be used to test for susceptibility, the other half to procreate. This strategy will insure the status of progenitors for further breeding experiments and the crossing of susceptible or resistant males to susceptible females will provide information on the contribution of the paternal genes to cowdriosis resistance and for the estimation of the heritability of characters linked to the resistance.

Breeding programs in cowdriosis-free areas in Guadeloupe have already been started. Since the Creole breed is very prolific, three generations per two years, results on the segregation of characters of resistance should be obtained in the near future.

DNA polymorphism and resistance to cowdriosis

Detection of very polymorphic regions in the genomes of the goats should provide an efficient tool to localize genes responsible for resistance to diseases. It is generally accepted that the location of 200 polymorphic markers equally spaced through the genome will provide a linkage map for any genome. Polymorphic markers could be genes such as the major histocompatibility complex or microsatellites (simple repeats spread throughout the genome whose length can vary). The establishment of linkage maps in several species, including sheep, has already been started and studies in this area will certainly help the research on genetic resistance to cowdriosis. The ultimate goal will be to correlate certain polymorphisms with resistance. The study of the segregation of polymorphisms in families from resistant and susceptible animal crosses should greatly assist in ascertaining whether a given region of the genome is linked to a gene involved in resistance to cowdriosis. Unfortunately, many polymorphisms detected by microsatellites in one species cannot be extrapolated to another species. However, it is possible that many microsatellite-associated polymorphisms will be conserved between goats and sheep.

Definition of the Creole goat genome is under way. Polymorphic regions such as the caprine major histocompatibility complex are currently being studied using oligonucleotides combined with polymerase chain reaction technology and genomic libraries targeting random sequences in the genome containing microsatellites will be performed.

The pathology of cowdriosis

Understanding the pathology of cowdriosis may help to explain the mechanisms of resistance in this disease. The pathology of this disease is very poorly understood but analysis of clinical symptoms between resistant and susceptible animals could direct research towards a more systematic investigation of what causes disease or death. The first observation is that resistant and susceptible animals are equally susceptible

to infection by *C. ruminantium;* the rickettsia is found inside neutrophils or brain capillary endothelial cells in both categories of animals. Most of the resistant animals undergo a brief febrile reaction while fever persists in susceptible animals. The second observation is that there is no correlation between rickettsaemia and death. Thus, mechanisms of growth control of the organism are not likely to be primarily involved in resistance. This would imply that *Cowdria* does not kill the animal but rather induces a severe metabolic change which results in the appearance of hydropericardium and hydrothorax which are more likely to be lethal. Also, the nervous symptoms are indicative of disorders affecting the permeability of the endothelial brain barrier. In the light of these observations, several metabolic pathways involved with the progression of the disease can be explored. The first question to be answered is what alterations does *C. ruminantium* induce in the host cell.

Bovine brain endothelial capillary cells can be cultured *in vitro* (Gospodarowski *et al.,* 1986) and passaged many times without alteration of their original phenotype. These cells can be readily infected with *C. ruminantium* (D. Martinez, unpublished data). Thus this system can be used to study the effect of infection on endothelial cells. Cytokine production can be monitored and compared between infected and non-infected cells. Endothelial cells produce interleukin 1 (IL1). This molecule has the capacity to activate T cells and, when over-produced, it is involved in causing fever and intestinal disturbances leading to diarrhoea. T-cell activation and hypersensitivity reactions resulting in inflammatory reactions are linked and could account for the pathology of cowdriosis. Other candidate molecules which can trigger inflammatory reactions are prostaglandins. Thus, it is possible that resistant and susceptible animals may differ in their ability to regulate cytokine and/or prostaglandin production. Therefore, the development of an *in vitro* system involving the culture of capillary endothelial cells from resistant and susceptible animals will be attempted in order to quantify cytokines, permeation factors and prostaglandins following infection with *Cowdria.* If differences are obtained, they should be confirmed in the animal itself.

A similar approach can be followed for neutrophils, although these cells are more difficult to maintain in culture for long periods. Unravelling the cascade of events which leads to the death of animals should therefore provide indications on the genes to target for detailed analysis in the families mentioned above.

CELL-MEDIATED IMMUNITY AND ITS POSSIBLE ROLE IN CONTROLLING COWDRIOSIS

In studies of the immunity against cowdriosis, it has been shown that antibodies are not protective and it has been suggested that cellular immunity is the most likely mechanism of protection (du Plessis, 1970, 1984). The mouse model, developed by du Plessis (1982, 1991), has shown that cell-mediated mechanisms play a key role in protection against cowdriosis and it is likely that this mechanism also provides protection in ruminants. Studies on the nature of immunity will lead towards better control of the disease through the development of safe vaccines.

The investigation of the cell-mediated immune responses against *C. ruminantium* will require breeding of goats and/or cattle to derive histocompatible animals

in which transfer of immunocompetent cell populations can be done. The CD8^{+} T-cell population, which comprises mainly cytotoxic cells, has been shown to provide protection against cowdriosis in the mouse (du Plessis *et al.,* 1991) and these cell populations will be studied in ruminants. Purification of ruminant CD8^{+} T cells could be achieved by lysing all other cells obtained from immune animals with monoclonal antibodies specific for the non-CD8^{+} cells (Howard *et al.,* 1991) and complement. These purified CD8^{+} cells will be transferred to histocompatible, naive animals previously infected with *C. ruminantium.* If the recipient animals survive infection, then a search for protective antigens, using the effector cell population, could be undertaken. Rickettsial genomes are relatively small (3.106 base pairs) and a systematic screening for antigens recognized by T cells is technically feasible. To be recognized by CD8^{+} T cells, antigens have to be synthesized inside the host cell and presented on the surface of the cell in conjunction with the major histocompatibility complex antigen. To achieve this, random genomic libraries will be constructed in appropriate vectors which should allow expression of foreign DNA in mammalian cells after transfection. Some vectors have the capacity to replicate in the cytoplasm of the host cell. Therefore on recognition of the specific antigen, CD8^{+} T cells will lyse the transfected cells liberating the plasmid in which genes of interest are inserted into the culture media. Development of such techniques will help in the identification of antigens which could be candidates for synthetic vaccines.

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Progress in development of subunit vaccines for anaplasmosis

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INTRODUCTION

Control of anaplasmosis can be achieved by vaccination with live and attenuated *Anaplasma* organisms (Henry *et al.,* 1983; Legg, 1936; Palmer, 1989; Theiler, 1912; Vizcaino *et al.,* 1980). Vaccines using killed *Anaplasma* organisms also induce adequate protective immune responses for disease control (Brock *et al.,* 1965; Montenegro-James *et al.,* 1990). The problem with the existing vaccines is that they are derived from the blood of infected calves. As a result, the most used attenuated vaccine is based on *A. centrale* organisms. However, only a few countries use this vaccine in control programs. Induction of a protective immune response in cattle with live, attenuated and killed *Anaplasma* organisms suggests that antigenically defined subunit vaccines can be developed. Subunit vaccines could be made by recombinant DNA or synthetic methods, possibly allowing widespread use.

PROTECTIVE IMMUNITY WITH NATIVE SURFACE PROTEINS

Our research on subunit vaccines for anaplasmosis was initiated by pursuing the hypothesis that isolated initial body surface proteins would induce a protective immune response against challenge with *A. marginale* organisms. This hypothesis was tested by identifying at least five initial body surface proteins (Palmer and McGuire, 1984), isolating three different surface proteins by monoclonal antibody affinity chromatography (Palmer *et al.,* 1986a, 1988b), immunizing calves with these proteins and challenging the calves with *A. marginale* organisms (Palmer *et al.,* 1985, 1986a, 1988a).

Surface proteins were identified using a strain of *A. marginale* isolated from cattle in Florida in 1955 (Ristic and Carson, 1977). Identification was by immunoprecipitation of proteins radioiodinated with a reaction catalyzed by lactoperoxidase (Palmer and McGuire, 1984). The primary antibody in the immunoprecipitation reactions was from immune sera that neutralized *A. marginale* initial body infectivity for cattle. Five of the Florida isolate surface proteins were designated by apparent molecular weights and included proteins of 105, 86, 61, 36 and 31 kilodaltons (Palmer and McGuire, 1984).

Following identification, the 105-kDa surface protein was shown to be a complex and designated major surface protein 1 (MSP1) complex (Allred *et al.*, 1990; Barbet *et al.*, 1983; Oberle *et al.*, 1988). The MSP1 complex was composed of protein heterodimers designated MSP1a and MSP1b. Monoclonal antibodies reacting with MSP1a were identified and designated ANA 22B1 or ANA 15D2 (McGuire *et al.*, 1984; Palmer *et al.*, 1986a). These monoclonal antibodies recognized a neutralization sensitive epitope on MSP1a that mapped to six amino acids of a tandem repeat of 29 amino acids in the MSP1a protein (Allred *et al.*, 1990). Variation in the size of the MSP1a protein in different *A. marginale* strains (Oberle *et al.*, 1988) was explained by the number of tandem repeats encoded in the 5′ end of the MSP1a gene of the strains (Allred *et al.* 1990). However, all strains examined, including nine from the United States, two from Kenya, two from Israel and one from Zimbabwe, express conserved epitopes which react with monoclonal antibodies 22B1 and 15D2 (McGuire *et al.*, 1984; Palmer *et al.*, 1988b; Tebele *et al.*, 1991). Also, these monoclonal antibodies bound to an epitope expressed on *A. centrale* organisms (Palmer *et al.*, 1988b). Therefore, the rationale for determining if the MSP1a complex could induce a protective immune response was based on the observation that MSP1a had a neutralization sensitive epitope conserved among all strains tested.

The native MSP1 complex was isolated from purified initial bodies of the Florida strain and disrupted with detergent (Palmer *et al.*, 1989, 1986a). Isolation was done with affinity columns containing either monoclonal antibody 22B1 or 15D2. In two different trials (Palmer *et al.*, 1989, 1986a), immunization of calves with MSP1 complex induced a significant protective immune response to challenge with the homologous strain of *A. marginale* when compared to calves immunized with a control antigen. Calf groups were immunized with either 50 or 100 µg of isolated MSP1 complex per injection mixed with Freund's complete adjuvant for the first injection and incomplete adjuvant for three subsequent injections. In each trial using five calves per group, two calves did not have microscopically detectable rickettsaemia or anaemia while the remaining calves had significantly lower rickettsaemias than two control groups immunized in the same way with ovalbumin and adjuvants (Palmer *et al.*, 1989, 1986a).

Immunization of calves with native MSP1 complex isolated from the Florida isolate of *A. marginale* also induced a protective immune response to challenge with heterologous Washington-O strain (Palmer *et al.*, 1989). In this case, none of five MSP1 complex immunized calves had detectable rickettsaemia or anaemia following Washington-O strain challenge while all five calves immunized with ovalbumin developed rickettsaemia. Rickettsaemias in the ovalbumin immunized and challenged

calves ranged from 3.6 to 5.5%. Packed cell volumes in these calves ranged from 25 to 28% compared to a range of 33 to 36% in the MSP1 complex immunized and challenged calves. The complete protection observed in the MSP1 complex immunized calves, evidenced by a lack of microscopically detectable rickettsaemia and anaemia following heterologous strain challenge, demonstrates that cross-protection against strains can be induced with isolated MSP1 complex (Palmer *et al.,* 1989).

MSP2, another surface protein of *A. marginale,* also induced a protective immune response in calves against both homologous and heterologous strain challenge (Palmer *et al.,* 1988b). The rationale for testing MSP2 was based on the presence of conserved epitopes among all strains tested from the United States, Kenya and Israel (McGuire *et al.,* 1984; Palmer *et al.,* 1988b) and on the positive results obtained with MSP1 complex. MSP2, originally described as a 36-kDa surface protein on initial bodies of the Florida strain (Palmer and McGuire, 1984), was isolated by affinity chromatography with monoclonal antibody ANAO 58A2 (Palmer *et al.,* 1988b). Surface exposed peptides of MSP2 from United States strains were conserved and the apparent molecular weights varied from 33 to 36 kDa. Groups of calves were immunized with either isolated MSP2 or ovalbumin using procedures similar to those described above for the MSP1 complex. Following immunization, calves were challenged with either Florida or Washington-O strains of *A. marginale.* Two of five MSP2-immunized calves did not have a detectable rickettsaemia following homologous challenge, two had a significantly delayed rickettsaemia, and one was not protected. Among five calves immunized with MSP2 and given a heterologous challenge, two did not have detectable rickettsaemia and the remaining three had very low rickettsaemias compared to the control group (Palmer *et al.,* 1988b).

A third surface protein, MSP3, is target for a subunit diagnostic test (McGuire *et al.,* 1991; Palmer *et al.,* 1986b) and was tested for its ability to induce a protective immune response against homologous challenge (T.C. McGuire, D. Knowles and G.H. Palmer, unpublished data). Initial designation of MSP3 as AmF86 (Palmer *et al.,* 1986b; Palmer and McGuire, 1984) was based on its apparent molecular weight of 86 kDa. MSP3 was isolated from the Florida strain by affinity chromatography using monoclonal antibody AMG 75C2 (McGuire *et al.,* 1991) and used to immunize calves. Five calves immunized with four injections of MSP3 in saponin had a significantly lower rickettsaemia when challenged than five calves given injections of saponin alone.

In conclusion, native MSP1a complex and MSP2 induce a protective immune response in calves against both homologous and heterologous challenge with *A. marginale*. These antigens are clearly candidates for subunit vaccine development.

PROTECTIVE IMMUNITY WITH MEMBRANE FRACTIONS

In addition to experiments on isolated surface proteins, tests of the hypothesis that an outer membrane fraction of *A. marginale* will induce protective immunity were done (Tebele *et al.,* 1991). Membrane fractions of isolated and sonicated initial bodies from the Norton Zimbabwe strain (Tebele and Palmer, 1991) were separated by sucrose density gradient centrifugation. A fraction banding at 1.22 g/cm^3 shared

epitopes with surface proteins of the Florida strain and contained outer membranes of *A. marginale* (Tebele *et al.,* 1991). Another membrane fraction that was distinct immunologically and by electron microscopy, banded at 1.15 g/cm^3. Immunization of cattle with either band suspended in saponin induced a protective immune response against homologous challenge with the Norton Zimbabwe isolate. When compared to control groups either not injected or injected with saponin alone, immunized groups had significantly less anaemia and significantly lower rickettsaemias. Four of five cattle immunized with the outer membrane fraction did not have microscopically detectable rickettsaemias, and protection in cattle immunized with both membrane preparations correlated with, and could be predicted with, antibody titre (Tebele *et al.,* 1991).

Identification of membrane proteins inducing a protective immune response may facilitate development of subunit vaccines either by being a component in a multiple subunit vaccine or being an effective single subunit vaccine.

RECOMBINANT AND SYNTHETIC REPLICAS OF PROTEIN SUBUNITS

Isolation of surface or other proteins from initial bodies derived from *A. marginale*-infected cattle blood is not a viable option for vaccine production. Therefore, we are now testing the hypothesis that recombinant and synthetic replicas of either the MSP1 complex or MSP2 will induce a protective immune response similar to that obtained with isolated native proteins. Genes from the Florida strain encoding the complete MSP1a and MSP1b proteins were cloned and sequenced (Allred *et al.,* 1990; Barbet *et al.,* 1983). Expression plasmids containing these genes were used to transform *Escherichia coli* and the individual expressed proteins were isolated by monoclonal antibody affinity chromatography (T.C. McGuire, G.H. Palmer and A.F. Barbet, unpublished data). Isolated recombinant proteins were mixed with saponin and used to immunize groups of calves. Even though calves developed high serum antibody titres to the respective native proteins, neither group had significant protection against challenge with the Florida strain when compared to a control group given saponin alone.

CURRENT RESEARCH

Several experiments are in progress and include those to evaluate complexes of recombinant MSP1a and MSP1b in order to more closely mimic native MSP1 complex, to make and test recombinant MSP2, and to further test synthetic peptides including the 29 amino acid tandem repeat of MSP1a.

ACKNOWLEDGEMENT

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Development of an antigenically defined vaccine against *Babesia bigemina*

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INTRODUCTION AND STRATEGY

The ability to induce immunity in cattle against experimental and field challenge with virulent *Babesia bigemina* is well established (Callow, 1977; Dalgliesh *et al.,* 1981). The most effective method of vaccination has been the inoculation of susceptible animals with live blood stage parasites that have been attenuated by passage in splenectomized calves (Callow, 1977; Dalgliesh *et al.,* 1981). Strong co-infectious immunity (premunition) induced in this way protects against both homologous and heterologous challenge (Callow, 1977; Dalgliesh *et al.,* 1981; de Vos *et al.,* 1982), although extensive testing of heterologous strain immunity under controlled conditions has not been performed. Equivalent induction of protective immunity has not been achieved with a crude killed antigen preparation (Callow, 1977; Kuttler and Johnson, 1980; Lohr, 1978; Young, 1988). Nevertheless, the ability of live blood stage parasites to induce homologous and heterologous protective immunity is an important observation, indicating that (i) antigens capable of inducing protection are expressed by blood stage parasites, (ii) cattle can respond to these antigens, (iii) conserved epitopes are present among geographically and antigenically distinct strains, and (iv) antigens expressed by blood stage parasites are capable of inducing protection against challenge with tick stages.

Despite the availability of an effective live vaccine, premunition has not been a widely adopted practice in areas where *B. bigemina* is prevalent. Drawbacks of this procedure include reversion to virulence, contamination of the vaccine with other blood-borne parasites, cumbersome production and quality control procedures involving live animals, necessity of maintaining a cold chain, and unavoidable contamination of the vaccine with bovine erythrocyte antigens (Callow, 1977). The history of vaccination against bovine babesiosis, current control procedures and epidemiology have been reviewed (Barbet 1989; Callow, 1977; Irvin, 1987; Taylor, 1989; Montenegro-James *et al.,* 1989; Goodger, 1989; Young, 1988; Wright and

Goodger, 1988; Ristic and Montenegro-James, 1988; Young *et al.,* 1988; Zwart and Brocklesby, 1979). The mechanism of immune protection in premunition is unknown. Passive transfer of immune serum confers significant protection against homologous challenge (Dwivedi and Gautam, 1980; Mahoney, 1967; Mahoney *et al.,* 1979). Non-B cell mediated mechanisms of immunity to babesial parasites have largely been unexplored. CD4 bearing T cells are important in generating T-cell help and amplification of the immune response and may, in conjunction with macrophages, elaborate soluble mediators such as gamma-interferon and tumour necrosis factor that have a direct effect on parasites. A role for cytotoxic T cells seems unlikely since the bovine erythrocyte does not appear to bear MHC class I molecules. Gamma-delta T cells may however be important in parasite elimination.

The general approach towards developing an improved method of immunoprophylaxis against *B. bigemina* has been to define the antigens responsible for the induction of protective immunity by blood stages. Based on accessibility to antibody and some subsets of T cells, antigens located on the surface of the merozoite and infected erythrocyte are likely targets of the protective response. Soluble parasite-derived mediators that have been described in *B. bovis* infection (Young, 1988) have not yet been characterized in *B. bigemina*-infected animals. However, since the destruction of erythrocytes in *B. bigemina*-induced clinical disease invariably exceeds the number of parasitized cells, and the osmotic fragility of uninfected cells from *B. bigemina*-infected animals is increased (Wright, 1973), it seems plausible that parasite derived or induced factors play a significant role in the pathophysiology of *B. bigemina* infection. These soluble antigens may also be targets of the protective response. Lastly, the unique apical organelles that define members of the Apicomplexa, including rhoptries and micronemes, by virtue of their putative role in invasion, may be a blockable step in the parasite's life cycle.

Initial studies have focused on merozoite surface antigens. The strategy for characterizing merozoite antigens with surface exposed epitopes was to identify monoclonal antibodies (MAb) that bound to the surface of intact, viable and infectious merozoites. These antibodies could then be used to identify, characterize and isolate merozoite polypeptides bearing surface exposed epitopes. The efficacy of individual antigens as protective immunogens could then be tested directly in an immunization trial, and the genes encoding surface proteins with neutralization-sensitive epitopes cloned and expressed. Recombinant genes expressing neutralization-sensitive epitopes would then be tested in different vaccine constructs, including vector-expressed antigens (such as vaccinia or related pox viruses, *Salmonella,* and others) with storage and administration properties amenable to use in tropical and subtropical areas of the world.

RESULTS

All studies have been performed with both uncloned and biologically cloned Mexican strains of *B. bigemina.* Infectious merozoites with intact cell membranes have been gradient separated in Percoll (McElwain *et al.,* 1987). Monoclonal antibodies (MAbs) that bound to the surface of these viable merozoites immunoprecipitate one of five apparently different ^{35}S-methionine-radiolabelled parasite

polypeptides or polypeptide complexes, with the major protein immunoprecipitated by different MAbs migrating in SDS-PAGE with apparent molecular weights of 72, 58, 55, 45, and 36 kDa. A MAb against the 58-kDa antigen also reacts with acetone-fixed infected cells in a patchy punctate pattern similar to MAbs against malarial rhoptry antigens in indirect immunofluorescent antibody assay (Clark *et al.,* 1987; Howard *et al.,* 1984; Perrin *et al.,* 1985; Peterson *et al.,* 1989; Roger *et al.,* 1988; Schofield *et al.,* 1986). Surface reactive MAbs identified by other investigators react with antigen complexes ranging in molecular weight from 68 to 36 kDa (Figueroa *et al.,* 1990), including the same 58-kDa antigen complex described above (unpublished data). Several of these MAbs are inhibitory to merozoite growth *in vitro* (Figueroa and Buening, 1991). Two additional MAbs have been identified that bind to the infected erythrocyte in acetone-fixed but not unfixed IFA assays. These MAbs immunoprecipitated an ^{35}S-methionine-labelled polypeptide of >200 kDa (McElwain *et al.,* 1989). All of these polypeptides appear to be recognized by antibodies in sera of cattle that are immune to challenge with *B. bigemina* (Figueroa *et al.,* 1990; McElwain *et al.,* 1987).

In vitro radiolabelling and immunoprecipitation studies indicate that post-translational modification of *B. bigemina* surface proteins is common (McElwain *et al.,* 1991). At least 10 parasite proteins are post-translationally modified by incorporation of glucosamine, while seven are myristilated. Five of the myristilated and glycosylated polypeptides can be immunoprecipitated by surface reactive MAbs. The 58-kDa antigen complex is not modified by the incorporation of glucosamine or myristic acid. It is interesting that most of the glycosylated and myristilated polypeptides of *B. bigemina* are known to be surface proteins. This does not appear to be the case with *B. bovis,* in which only one known surface polypeptide will incorporate glucosamine or myristic acid (Hines *et al.,* 1989).

Initial studies of cross-reactivity of MAbs with geographically different strains of *B. bigemina* in immunofluorescence assays indicated that MAb epitopes on gp55, gp45, and gp36 were variant. More extensive immunoblot studies using monospecific rabbit and bovine antisera prepared against purified antigens confirmed that epitopes on gp45, gp55, and gp36 were not well conserved among geographic isolates. When cross-reactivity was present, most isolates expressed polypeptides of approximately the same molecular weight. The exception was that MAb against a Mexican isolate gp45 and gp55 polypeptides identified high molecular weight polypeptides in a Kenya isolate of *B. bigemina.* In contrast to these results, the p58 surface polypeptide/rhoptry antigen was conserved in all isolates tested, and always migrated with the same or nearly the same molecular weight in polyacrylamide gels. Additional MAbs identified by other investigators have been widely cross-reactive. All these results have been previously published or are in press (Figueroa *et al.,* 1990; Hines *et al.,* 1989; McElwain *et al.,* 1987, 1988, 1991), and are summarized in Table 1.

To test the efficacy of these merozoite surface proteins as immunogens, groups of five cattle were immunized with immunoaffinity chromatography purified 58, 55, 45 or 36 kDa antigens, or a control protein (ovalbumin) in Freund's adjuvant. The purity and specificity of the antigen preparations were confirmed by silver-stained SDS-PAGE and Western immunoblots, respectively. All cattle were challenged with 3×10^9 uncloned, Mexican strain-infected erythrocytes from a splenectomized donor

TABLE 1. *Babesia bigemina* surface-exposed epitopes.[*]

Polypeptide (kDa)	Epitope location	Isolate conservation	Comments
36	merozoite surface	Mexico, Texcoco, St. Croix	glycosylated/ myristylated
45	merozoite surface	Mexico, Kenya	glycosylated/ myristylated
55	merozoite surface	Mexico, Texcoco, Kenya only	glycosylated/ myristylated
58	merozoite surface	All tested to date	putative rhoptry
72	merozoite surface	N/A	
200	parasitized rbc	N/A	

[*] Isolates tested to date include the American isolates (Mexico, Texcoco, Puerto Rico, St. Croix), Israel isolate and an African isolate (Kenya). Correlation with monoclonal antibodies binding to Costa Rica, Texas and Tecocutla isolates is pending.

calf. Animals immunized with p58, gp55 and gp45 were able to neutralize the infectivity of merozoites as judged by a statistically significant reduction in the peak parasitaemia as compared to controls. The severity of clinical disease in these groups was also reduced, as 4/5 controls but 0/5 gp45 immunized cattle and 1/5 p58 and gp55 immunized cattle had haemoglobinemia and haemoglobinuria. Percentage reductions in PCV and rises in rectal temperature were reduced in the gp45, gp55 and p58 immunized groups in comparison to controls, but these changes were not statistically significant on a group basis due to an outlier effect. These results are summarized in Table 2 (McElwain *et al.,* 1991).

The gene encoding the merozoite surface and putative rhoptry protein, p58, has been cloned, expressed, sequenced and characterized. Recombinant protein expressed by the cDNA copy induces antibodies in rabbits that immunoprecipitate native p58, and bind to the surface of live merozoites from all strains tested. A minimum of four gene copies are detected in Southern blots of genomic DNA from a cloned Mexican strain of *B. bigemina.* Four different copies of the gene encoding p58 have been cloned from polymerase chain reaction (PCR)-amplified DNA using primers flanking the long open reading frame. All four copies have been completely sequenced and compared. There are substantial blocks of sequence divergence located in both the 5' and 3' halves of the protein. In comparison to the gene copy encoding the cDNA expressed polypeptide, one of the four genes contains a divergent 3' region, one has a divergent 5' region, and one has both a 3' and a 5' divergent regions that are present in the other two divergent gene copies. Using gene specific probes on PCR-amplified DNA reverse transcribed from mRNA, it can be shown that three of the four gene copies are simultaneously expressed in the cloned strain. Further characterization of the p58 gene copies and the significance of the sequence divergence to function are being investigated. A partial copy of the gene encoding gp55 has also been cloned and expressed, but has not been further characterized (Mishra *et al.,* 1991).

TABLE 2. Immunization of cattle with purified *Babesia bigemina* merozoite surface proteins

Immunogen	Animal	ELISA titre	Surface IFA titre	Peak % parasitemia	Group Peak % parasitemia[*]
Ovalbumin	B258	—	—	2.5	
	B257	—	—	1.7	
	B263	—	—	2.4	1.8 +/–0.7
	B262	—	—	0.8	
	B235	—	—	1.4	
gp45	B264	5×10^4	10^2	0.6	
	B260	10^4	10^2	0.6	
	B265	10^4	10^2	0.9	0.7 +/–$0.1^{\dagger}$
	B256	10^4	10^2	0.7	
	B261	5×10^4	10^2	0.6	
p58	B279	10^4	10^2	0.3	
	B277	10^4	10^2	0.9	
	B274	10^4	10^2	1.2	0.8 +/–$0.4^{\dagger}$
	B273	5×10^4	10^2	0.9	
	B272	5×10^4	10^2	0.5	
gp55	B271	10^4	10^1	0.6	
	B275	10^3	10^1	0.6	
	B276	10^4	10^2	0.5	0.8 +/–$0.6^{\dagger}$
	B278	10^4	10^2	0.6	
	B238	10^4	10^2	1.9	
gp36	B266	10^4	10^1	1.8	
	B267	10^4	10^1	1.7	
	B268	10^3	10^1	2.0	1.4 +/–0.6
	B269	10^4	10^1	0.4	
	B270	10^4	10^1	1.2	

[*]mean +/–standard deviation
[†]significantly different from ovalbumin controls, $p < 0.05$

CONCLUSIONS

While our results indicate that an immune response directed against individual surface exposed merozoite antigens can neutralize their infectivity, it is clear that isolated native merozoite surface proteins do not induce sufficient protection against clinical babesiosis. In our trial, only a small portion of the antibody response was against the surface of the merozoite (Table 2). Directing the response towards specific surface exposed epitopes may enhance the efficacy of these antigens. However, induction of immunity against clinical babesiosis may require a combination of antigens that target the response towards all surfaces or molecules

for which a protective response may be effective or a pathophysiologic effect neutralized. In addition, the method of antigen presentation may be important, and antigenic diversity or variation may limit the usefulness of some otherwise effective antigens. Thus, we are directing our research towards (i) testing of the conserved merozoite surface protein p58 and the high molecular weight erythrocyte membrane protein as immunogens alone and in combination, (ii) defining the polypeptides present on the infected erythrocyte surface and in the apical complex organelles, and (iii) testing the efficacy of expression vectors for presentation of antigens.

ACKNOWLEDGEMENTS

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Vaccination against *Babesia bovis* using antigens produced by recombinant DNA technology

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INTRODUCTION

The tick-transmitted haemoprotozoan parasite *Babesia bovis* is the causative agent of the most severe form of bovine babesiosis (tick fever) and is responsible for major economic losses in the tropical and sub-tropical cattle industries of Africa, Latin America, Australia and South East Asia. After infection with *B. bovis,* recovered animals remain sub-clinically infected and immune for life. In *Babesia*-endemic areas calves receive maternal antibody from immune mothers which confers protection against *B. bovis* challenge in early life (Hall, 1960, 1963). *Babesia bovis* mortality in young, immunologically naive animals is far lower than in adult cattle. Greatest mortality occurs in areas where tick-transmission of *B. bovis* is highly unstable; those areas bordering endemic regions or in instances where animals are moved from tick-free to tick-infested areas.

Current strategies for the control of *B. bovis* include the extensive use of acaricides to control ticks and the use of a live attenuated vaccine developed and produced commercially in Australia (Callow *et al.,* 1979). Vaccination against *B. bovis* using a culture-derived antigen preparation (Smith *et al.,* 1981; Montenegro-James *et al.,* 1989) has been extensively tested in Venezuela, although the efficacy of this vaccine, particularly with regard to cross-strain protection, is questionable (Timms *et al.,* 1983). Although preimmunization using the live attenuated vaccine confers a high degree of protection, a number of disadvantages are inherent with its production and use. The vaccine is produced in splenectomized calves and contamination with other pathogens may occur (Wright and Riddles, 1989). A proportion (approximately 5%) of vaccinated animals react severely to infection with the attenuated parasite and require drug treatment to prevent mortality. All animals show a significant haematocrit fall (mean value approximately 30%) upon vaccination and retain the potential to transmit the parasite (which may revert to virulence) to other animals. Some problems with strain variation in the field and subsequent breakthrough of infection have been observed (A.J. de Vos, personal communication).

The involvement of the CSIRO in research on babesiosis dates back to the work of Mahoney in 1962 which demonstrated that immunization using killed whole parasite antigen induced a high level of protective immunity (Mahoney, 1967; Mahoney and Wright, 1976) and that serum from immune animals conferred protection against *B. bovis* infection when transferred into susceptible, infected animals (Mahoney *et al.,* 1979). The humoral immune response plays an important part in immunity to *B. bovis* and vaccination with exogenous antigen is a feasible approach to vaccine production. More recent work has implicated immune effector cells in addition to antibody as an important mechanism of immunity to *B. bovis* (James, 1988; Jacobson *et al.,* 1991).

FRACTIONATION AND TESTING OF *BABESIA BOVIS* ANTIGENS IN VACCINATION TRIALS

The approach taken by our group has been to systematically fractionate the material obtained by disruption of *B. bovis*-infected erythrocytes using a variety of techniques including centrifugation, precipitation, electrophoresis and various types of chromatography. At every stage of purification, each fraction was tested in a vaccination/challenge experiment to assay for the potential to induce protective immunity. Protective antigen fractions were used to raise monoclonal antibodies (MAbs), which were then tested for reactivity with babesial antigen by Western blotting and indirect immunofluorescence test (IFAT) against *B. bovis*-infected erythrocytes. Monoclonal antibodies were then used to select babesial antigens which were purified by affinity chromatography and the purified antigens tested in vaccination experiments. Single antigens which induced protective immunity were then produced using recombinant DNA technology.

THE W11C5 ANTIGEN

Of eight *B. bovis* antigens that were purified from the `β` protective fraction (Goodger *et al.,* 1985) by MAb affinity chromatography, one was found to induce protective immunity in vaccinated animals challenged with virulent *B. bovis* parasites of strains both homologous and heterologous to the strain used for antigen purification. This antigen was designated W11C5 after the nomenclature of the MAb reactive with it. The W11C5 MAb reacted predominantly with the *B. bovis*-infected erythrocyte membrane in IFAT and produced a stepladder of bands on Western transfers of `β` antigen from approximately 70 to > 200 kDa. The W11C5 MAb was used to screen a cDNA expression library constructed in the vector λgt11 (Huynh *et al.,* 1985). Of ten positive clones examined, one was found to express a large beta-galactosidase fusion protein of approximately 280 kDa (β-gal = 120 kDa). The W11C5 fusion protein was reactive with the W11C5 MAb on Western transfers and produced a stepladder of discrete break-down products similar to that evident on Western transfers of `β` antigen probed using the MAb. The W11C5 fusion protein, which comprised 1–2% of total soluble *Escherichia coli* protein, was tested in vaccination experiments as SDS-PAGE

gel-band purified material and as unpurified soluble *E. coli* lysate at a dose of 10 μg per animal per vaccination in a standard two-shot, four-week interval vaccination regime using Freund's complete adjuvant (FCA). Sera from the vaccinated cattle were shown to react in IFAT with the *B. bovis*-infected erythrocyte membrane and produced a stepladder of bands on Western transfers of β fraction antigen and W11C5 fusion protein characteristic of W11C5 MAb reactivity. Upon challenge with heterologous strain *B. bovis*-infected erythrocytes, animals vaccinated with the W11C5 fusion protein, either SDS-PAGE purified or as crude *E. coli* lysate, developed significantly lower parasitaemias (mean maximum 74 ± 27/μl and 479 ± 424/μl respectively) and exhibited increased survival rates (5/6 and 6/6 respectively) relative to two groups of control animals vaccinated with the corresponding beta-galactosidase only materials (mean maximum 3609 ± 2509/μl, 1/6 survival and 6725 ± 3327/μl, 0/6 survival, respectively). Expression and purification of the W11C5 recombinant antigen was enhanced by cloning into the plasmid vector pGEX-1 (Smith and Johnson, 1988). This yielded an expression greater than 30% of total *E. coli* protein of a soluble W11C5-glutathione S-transferase (GST) fusion protein which was purified in a single step by affinity chromatography using glutathione-agarose. The purified W11C5-GST fusion protein was found to induce levels of immunity to *B. bovis* similar to that obtained using the beta-galactosidase fusion protein without the pyrogenic effect of contaminating *E. coli* lipopolysaccharide (LPS). In addition, the W11C5 antigen was found to be as effective when used with the commercially acceptable adjuvants Quillaja saponin of Quil A as with FCA.

ORGANIZATION AND STRUCTURE OF THE W11C5 GENE

Analysis of the expression and organization of the W11C5 gene using the W11C5 cDNA as a hybridization probe demonstrated that the full length transcript of the W11C5 gene is approximately 10.0 kb (hence encoding a complete protein of approximately 350 kDa) and that the W11C5 gene is present as a single copy in all strains examined including several Australian and one African isolate. The W11C5 gene homologues have not been detected in *B. bigemina* or *B. canis* or in *Theileria* species, although immunological cross-reactivity of the W11C5 MAb has been reported with *B. ovis* and *B. divergens* (I.G. Wright, personal communication).

DNA sequence analysis has shown that the W11C5 cDNA (3.1 kb) is composed of two distinct regions—a 5' repetitive region consisting of 19 copies of a 90 base pair (bp) sequence and a 3' unique sequence region. The repetitive and unique regions were separately subcloned and expressed as GST fusion proteins for testing in vaccination experiments. The purified W11C5 repetitive region fusion protein was found to induce no protection to *B. bovis* challenge whereas the W11C5 unique region fusion protein was protective. The failure of the repetitive region to induce protection provides evidence to support the hypothesis that the highly immunogenic repetitive antigens commonly found in parasites exposed to the host immune system (Kemp *et al.,* 1987) have evolved to act as immunological smoke-screens (Anders, 1986) or are immunosuppressive (Liew *et al.,* 1990). The repetitive region of the W11C5 antigen may represent a built-in smoke-screen or immunosuppressive domain which functions to decrease the immune response of the

host to the unique region of the antigen which may be a functionally important protein domain. The observation that immunodominant antigens do not generally induce a protective immune response has been confirmed by our group with regard to a purified *B. bovis* antigen (Goodger *et al.,* 1986) and in fact these antigens may abolish the protective potential of other antigens used concurrently in a mixture of immunogens (I.G. Wright and K.R. Gale, unpublished results).

THE 12D3 ANTIGEN

The MAb 12D3 is a more stable IgG1 subclass antibody producing clone reactive with the 15B1 protective antigen (Wright *et al.,* 1983), originally isolated from a protamine sulphate precipitated antigen fraction. The 12D3 MAb was shown to react by IFAT with the *B. bovis* parasite and the infected erythrocyte cytoplasm and on Western transfers of *B. bovis* antigen with a 38-kDa soluble antigen. A cDNA clone containing the entire coding sequence of the 12D3 antigen was isolated from a λgt10 cDNA library (Huynh *et al.,* 1985) using a DNA probe designed using the N-terminal amino acid sequence of the purified 12D3 protein. Recombinant 12D3 was expressed in *E. coli* as highly insoluble inclusion bodies which required the addition of SDS and 2-mercaptoethanol to achieve solublization. Vaccination of cattle using renatured 12D3 recombinant antigen resulted in a significant reduction in parasitaemia upon *B. bovis* challenge relative to control animals, although the level of protection was not as high as was obtained using the native *B. bovis* expressed protein (Riddles *et al.,* 1990). DNA sequence analysis of the 12D3 gene revealed a very high cysteine residue content (approximately 10%), a signal peptide, indicating that the protein is transferred across a membrane, and the potential to form epidermal growth factor-like domains (Riddles *et al.,* 1991). No repetitive sequences have been identified within the 12D3 gene. Due to the high cysteine content it is probable that the conformation of the 12D3 protein as produced in *E. coli* and after denaturation and renaturation *in vitro* is not representative of the native 12D3 protein. Further work to enhance the protective potential of this antigen by expression in a eukaryote system (baculovirus) is currently under way. DNA hybridization studies have revealed that the 12D3 gene is conserved across a range of different species of *Babesia*. Homologues of the gene have been detected in *B. bigemina, B. canis, B. bovis* and *B. divergens*. The 12D3 homologue from *B. bigemina* has now been cloned and sequenced and is approximately 60% similar to the *B. bovis* gene at the amino acid sequence level (P.W. Riddles, in preparation).

VACCINATION TRIALS USING RECOMBINANT ANTIGEN COCKTAILS

To investigate the effect of combining recombinant protective antigens, the W11C5 and 12D3 antigens were used in combination in a vaccination trial. Together, the antigens induced a 99% suppression of parasitaemia in the vaccinates relative to the control animals. Both parasite suppression and the suppression of fall in haematocrit value were superior to the results obtained using the antigens singly. When this antigen combination was tested under field trial conditions, 10% of vaccinated

animals required treatment compared to 40% of the control animals. Mean maximum parasitaemias were $232 \pm 345/\mu l$ in the vaccinates and $2154 \pm 3065/\mu l$ in the controls. Mean maximum haematocrit falls were $30.11 \pm 14.74\%$ and $45.50 \pm 10.61\%$ for the vaccinates and controls respectively.

Although the W11C5/12D3 antigen cocktail induced 90% protection under field trial conditions, it was decided to add a third recombinant antigen to the cocktail in an attempt to further improve vaccine efficacy. The antigen selected, designated T21B4, was recently cloned using the MAb of the same designation (R.E. Casu, unpublished results) which was raised against the `protease' protective antigen fraction (Commins *et al.,* 1985) and shown to induce protection in a preliminary vaccination trial. The W11C5/12D3/T21B4 antigen cocktail was tested in a field trial in direct comparison with the Queensland Department of Primary Industries (QDPI) live attenuated vaccine. Under conditions of heavy tick infestation and coincidental *B. bigemina* challenge, the trivalent antigen vaccinates (n = 18) underwent a mean maximum fall in haematocrit value of $16.02 \pm 3.30\%$, a mean maximum parasitaemia of $824 \pm 34.8/\mu l$ and a 0/18 treatment rate compared with the naive controls (n = 19) which showed a mean haematocrit fall of $35.95 \pm 2.95\%$, a mean maximum detected parasitaemia of $6962 \pm 152.5/\mu l$ and a treatment rate of 13/19. Upon vaccination, the QDPI group (n = 40) showed a mean maximum fall in haematocrit value of $25.82 \pm 3.13\%$, a mean maximum detected parasitaemia of $1206 \pm 3939.58/\mu l$ and 2/40 animals required drug treatment. Upon challenge with *B. bovis,* the QDPI vaccinates showed a mean maximum fall in haematocrit value of $19.91 \pm 1.72\%$, a mean maximum detected parasitaemia of 141 ± 27.0 and a treatment rate of 1/38. Further vaccination trials are currently under way to test the efficacy of the trivalent vaccine under a range of field challenge conditions. The feasibility of developing vaccines against other species of *Babesia* using the existing protective antigens or their homologue clones from different species is currently being investigated. The natural diversity observed in protozoan parasite species (Kemp and Cowman, 1990) and the ability of most species to rapidly generate genetic diversity by either somatic mutation/recombination or recombination during sexual reproduction (Walliker, 1989) poses a serious threat to the success of any vaccination strategy. This problem is likely to be enhanced if a recombinant vaccine containing a single antigen is used. For this reason it is proposed to pursue the development of a vaccine based on several distinct protective antigens, each of which induces significant protection in the vaccinated host. The analysis of strain variation by analysis of restriction fragment length polymorphisms (RFLP) in all Australian strains used during the work of this group has been reported (Dalrymple *et al.,* 1992). Investigation of future changes in Australian *B. bovis* population composition in response to vaccine use is considered by our group to be essential to ensure long-term success of a recombinant vaccine. The polymerase chain reaction (PCR) (Saiki *et al.,* 1988) will provide a powerful tool with which to determine directly the extent of sequence polymorphism in the vaccine antigen genes of field isolates, thus enabling appropriate changes in vaccine composition to be made if necessary.

SUMMARY

We have found that *B. bovis* immunodominant antigens, which are preferentially cloned when expression libraries are screened using immune sera from previously infected animals, do not induce a protective immune response in the host. The approach we have used of systematic fractionation of parasite antigen, testing of each fraction in vaccination trials and progressive purification to yield single components with the ability to induce protective immunity in cattle, although painstaking, has proven successful. To achieve protection comparable with that obtained using a live *B. bovis* vaccine or killed antigen preparations, several distinct recombinant antigens are probably required. A recombinant vaccine against *B. bovis* should provide a safer, more commercially acceptable alternative to the live attenuated vaccine currently used in, and exported from, Australia.

ACKNOWLEDGEMENTS

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Identification of *Babesia divergens* protective antigens

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INTRODUCTION

Babesia divergens, a tick-transmitted haemoprotozoa, is the main agent of bovine babesiosis in Europe (Kuttler, 1988) and is responsible for important economic losses in cattle. Unlike other *Babesia* species, *B. divergens* infects not only cattle but other mammalian species such as man (Gorenflot and Brasseur, 1991) and gerbils. In Europe, several human infections have been recorded especially in splenectomized patients, and a high mortality rate has been reported. In attempts to protect cattle from clinical disease, different vaccination strategies have been investigated using live vaccines (Dalgliesh *et al.,* 1990; Wright *et al.,* 1982) or inactivated vaccines (Goodger *et al,.* 1984; Montenegro-James *et al.,* 1987; Moreau *et al.,* 1988; Ristic and Monte-negro-James, 1988; Winger *et al.,* 1987). Live vaccines, attenuated parasites or irradiated parasites provide good protection but have several restricting features: a short shelf life, a possible reversion to virulence, the risk of transmission of other pathogenic agents and the maintenance of healthy carriers. Identification and charac-terization of protective antigens make it possible to avoid some of these shortcomings,

although the development of an industrial vaccine requires a safe, potent and well characterized product which is easy to produce and standardize. The development of methods for *in vitro* propagation of *Babesia* provides a useful technique to obtain and characterize antigens involved in protective immunity.

IN VITRO CULTURE

A major breakthrough in babesiosis research was achieved by the development of the microaerophilus stationary phase (MASP) method for *in vitro* propagation of *B. bovis* (Levy and Ristic, 1980). When applied to *B. divergens,* this method was reported to give parasitaemia levels lower than 15 percent. In contrast, our long-term *in vitro* culture (Gorenflot *et al.,* 1991) of more than 305 subpassages of *B. divergens* in human red blood cells routinely exhibited parasitaemia levels of about 30 to 40 percent. This high parasitaemia was not a feature of only the original isolate but all European isolates tested could infect human erythrocytes and produce high parasitaemia with only slight differences in the growth rates. Furthermore cultures could be initiated from human, bovine or gerbil erythrocytes without any marked differences and, after a few passages, the original erythrocytes disappeared and the isolate was stabilized. The optimal conditions of our culture required a serum concentration lower than those used in previous studies and after more than two years of continuous *in vitro* culture, there is no apparent modification of the virulence when tested in gerbils.

EXPERIMENTAL TRIALS ON GERBILS

In order to evaluate immunogenicity of *in vitro* culture-derived antigens, we prepared a vaccine for evaluation in gerbils, a convenient laboratory model for *B. divergens* infection (Gorenflot *et al.,* 1990). One hundred gerbils were randomly divided into five equivalent groups and immunized with different doses of parasitized culture supernatant equivalent (PCSE) emulsified with 0.2 mg of Quil A. Control groups received 1.5 ml of unparasitized culture supernatant equivalent (UPCSE) plus Quil A or saline solution alone. After homologous challenge, the gerbil mortality rate in control groups varied from 70 to 90 percent. In contrast, all gerbils vaccinated with two or three injections of 1.5 ml or 0.3 ml of PCSE survived, except one animal vaccinated twice with 0.3 ml of PCSE. In the group receiving 0.06 ml of PCSE, the mortality rate was similar to the control groups.

Antibodies in gerbils vaccinated with the full vaccine (1.5 ml of PCSE) or a 1:5 diluted vaccine dose (0.3 ml PCSE) were measured using an immunofluorescence assay. In gerbils vaccinated with a full vaccine dose, antibodies were observed 14 days after the first injection. A second or third injection of vaccine did not strongly enhance the antibody titres. Comparatively, only the third injection of the 0.3 ml PCSE vaccine dose was able to induce antibody titres as high as those obtained with only one injection of a full vaccine dose. On day 17 after challenge, the antibodies reached similar levels in all gerbils.

Analysis of the antigenic specificities of the antibody responses was performed by immunoprecipitation. *Babesia divergens* antigens radiolabelled with [35]S-

methionine were immunoprecipitated with sera from gerbils vaccinated with one, two or three injections of 1.5 ml of PCSE before and after challenge. The first injection of 1.5 ml of PCSE induced a strong humoral response against several *B. divergens* antigens. In contrast with results obtained by the immunofluorescence assay, some differences were observed after the second and third injections: two bands of molecular weight of about 40 kDa after the second injection and two new bands after the third injection, one of high molecular weight, the other of low molecular weight. Virulent challenge did not significantly modify the antibody pattern obtained with vaccination.

In the second trial, a vaccination protocol was designed to assess the protection provided by *B. divergens in vitro* culture supernatant against heterologous challenge with different geographical isolates (Precigout *et al.,* 1991). Gerbils were vaccinated twice with 1.5 ml of PCSE mixed with 0.2 mg of Quil A on days 0 and 21. Controls received 1.5 ml of UPCSE plus Quil A. Three weeks after the last injection, vaccinated animals and controls were divided into four groups. Each group was challenged with different isolates: French homologous isolate, French heterologous isolate, German isolate and UK isolate. On challenge, the gerbils were fully protected against the two French isolates and partially protected against the UK (80 percent) and the German isolates (60 percent). The differences observed could be due to the variable virulence of the isolates. Indeed, the UK and the German isolate were significantly more virulent (100 percent mortality in control groups) than the French isolates (80 to 90 percent of mortality rate in control groups). In addition the mortality was more rapid with the UK and German isolates than with the French isolates.

BOVINE IMMUNIZATION

To confirm the validity of the gerbil model for vaccine assessment, we have conducted a limited trial in cattle. The first step was to define the optimal conditions for this animal model to be able to induce sufficient disease to discriminate between the protected and unprotected animals. After several experimental infections, we concluded it was very difficult to induce a severe and reproducible infection in intact animals, so splenectomized cattle were used.

Four 18-month-old cattle, bred in a tick-free area, were splenectomized and divided into two groups of two animals each. The first group received 15 ml of PCSE emulsified with 1.5 mg of Quil A. The immunogens were injected on days 0, 21 and 42. The second group received 15 ml of UPCSE plus Quil A. Three weeks after the last injection all the animals were challenged with 3×10^{10} fresh parasitized bovine erythrocytes intravenously. Protection was assessed on the basis in the level of parasitaemia and reduction in packed cell volume (PCV).

Parasites were detected in the two controls on day 2 after challenge. In contrast one of the vaccinated calves had detectable parasites on day 2 and the second on day 4. Parasites were observed in all animals, but a significant difference was seen between the daily parasitaemia of vaccinates and controls. In one of the controls, parasitaemia increased very rapidly to reach 30 percent on the seventh day after challenge. In the other control the parasitaemia reached 2.2 percent. In contrast, the parasitaemia in the vaccinates did not exceed 0.7 percent. The clearance of the

parasites was also faster in the vaccinates than in the controls. All cattle had a reduction in PCV but the maximum percentage reduction was significantly lower in vaccinates than in controls. Maximum PCV reduction in one control was 71 percent on day 6 after challenge and 44 percent on day 11 in the other. In the vaccinated calves the maximum PCV reductions were 19 and 24 percent, respectively. In addition the return to normal PCV was rapid in vaccinates (day 14 and 20). The PCV did not return to normal for seven weeks after challenge in the controls.

In an attempt to combine the PCV reduction and the time to return to normal PCV as a single parameter, we developed a new criterion: the cumulative PCV reduction. This was calculated using the area delimited by the line of the 0 percent reduction and the curve of the daily relative PCV until the return to normal value. With this criterion, the difference between controls and vaccinates was very marked: the cumulative PCV reduction in the controls was four times greater than that for the vaccinates.

The serological response after vaccination was estimated using an IFA test. The antibody titre was 1/400 after the first vaccine injection and 1/1200 after the second injection. The third injection did not increase the IFAT antibody and at the time of challenge the two vaccinate animals had antibody titres of between 1/1200 and 1/1600.

In conclusion, the experiment in cattle confirmed that a crude *B. divergens in vitro* culture supernatant could induce good protection in splenectomized cattle. This crude supernatant appeared to be a good source of material from which to identify and purify antigens involved in protection. In addition the gerbil seemed to be a good laboratory model for screening antigens involved in protection.

CHARACTERIZATION OF PROTECTIVE ANTIGENS

In order to facilitate identification and purification of the antigens involved in the protection, we developed a semi-defined medium which supported the growth of *B. divergens* without serum. The multiplication rates and the levels of parasitaemia obtained with this medium were similar to those observed with the classical medium. Exoantigens obtained using this new culture method were concentrated and emulsified with saponin and conferred complete protection in gerbil to homologous challenge.

Several defined fractions from this aseric culture supernatant were purified by chromatography. These fractions were mixed with saponin and administered subcutaneously in three doses on days 1, 21 and 42. After challenge with the homologous isolate, one of these fractions conferred a very strong immunity with no mortality in vaccinated animals. The immunity induced by this fraction was similar to that observed with total supernatant.

A protein bearing an epitope in this defined protective fraction recognized by monoclonal antibody (MAb) DG7 was identified (E. Precigout, A. Valentin, B. Carcy, A. Gorenflot, K.I. Nakamura, M. Aikawa and J. Schrevel, unpublished data). The monoclonal antibody labelled the whole parasite membrane in different stages but, in merozoites, an intracellular and linear structure was clearly recognized. After disruption of purified merozoites, the MAb DG7 staining was detected not only on

the merozoite membrane but also on a released tubular structure, indicating a possible organelle location. Similar staining was observed with different *B. divergens* isolates and with *B. canis* in acetone fixed smears. As the immunofluorescence localization of the protein bearing the DG7 epitope seemed to be restricted predominantly to *B. divergens* membranes, a Triton X 114 phase separation was performed followed by an immunoprecipitation by MAb DG7. The protein bearing epitope DG7 was evidenced only in the aqueous phase and never in the detergent phase or in the detergent insoluble pellet. Immunogold labelling of *B. divergens*-parasitized erythrocytes was localized along the peripheral cytoplasm just beneath the membrane of the merozoite. The sub-membrane localization observed with immunogold, and the hydrophilic character of this protein demonstrated by Triton X 114 phase separation, could indicate that the protein bearing the MAb DG7 epitope is an extrinsic protein present on the cytosol side of the membrane. Purified MAb DG7 specifically inhibited the growth of *B. divergens in vitro* at very low concentrations. The inhibitory concentration 50 percent (IC50) was estimated to be 26 µg/ml after 17 hours, 18 µg/ml after 26 and 16 µg/ml after 42.

CONCLUSIONS

The inhibitory effect of the MAb DG7 on the growth of *B. divergens in vitro,* the demonstration that the protein bearing the epitope is a major constituent of a chromatographically defined fraction of *B. divergens in vitro* culture supernatant which confer complete homologous protection in gerbils, the distribution of this antigen in different geographic isolates of *B. divergens* and other *Babesia* species indicate that this protein could be a good vaccine candidate against the asexual blood stage of *B. divergens.*

ACKNOWLEDGEMENTS

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IN VITRO CULTIVATION

Cultivation of *Babesia*

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Babesiosis is a tick-borne intra-erythrocytic protozoan parasite which causes a disease of domestic and some wild animals characterized by fever, haemolytic anaemia, haemoglobinuria and death. From an economic point of view, bovine babesiosis is a serious problem as it is widely distributed and threatens the health and safety of some 500 million cattle in tropical and semi-tropical regions of the world. Eighteen of the many species of *Babesia* cause disease in domestic mammals, man and laboratory animals, the four economically important bovine species being *B. bovis, B. bigemina, B. divergens* and *B. major.* The tropical species *B. bovis* and *B. bigemina* often occur together and in association with *Anaplasma marginale* and are transmitted by the same tick species.

Initially the culture of *Babesia* spp. was seen as a source of antigens for immunological studies and for improved vaccine. However, the continuous growth of *Plasmodium falciparum in vitro* (Trager and Jensen, 1976) has clearly demonstrated the wide range of experimental uses for such cultures in addition to the primary interest in vaccines. These include basic biological/biochemical investigations, invasion mechanisms, host and strain specificity, cloning of parasites, virulence factors, interaction with the immune system and chemotherapy assays.

The feasibility of adapting the method for culturing *P. falciparum in vitro* (Trager and Jensen, 1976) to *Babesia* was first shown by Erp *et al.* (1978). Short-term *in vitro* multiplication of *B. bovis* was obtained using suspensions of bovine erythrocytes incubated at 38 °C in medium-199 with 50% bovine serum, and a gas phase of 5% CO_2 in air. The erythrocytes were maintained in suspension by slow stirring (100 rpm) in spinner flasks and the medium was replaced at 24-hour intervals. Parasite multiplication did not continue beyond three days, but this system was used to determine the most important parameters for long-term growth. With minor adaptations, continuous growth was eventually obtained (Erp *et al.,* 1980a) and further shown that parasites cultured for up to 85 days were still able to infect a splenectomized calf. The important culture parameters were identified as the use of freshly collected erythrocytes at a packed cell volume (PCV) of 20–30%, anticoagulation of blood by defibrination, complex tissue culture media such as medium-199, NCTC-135 or RPMI-1640, stringent control of the pH of the medium (pH 7.0) for which 25 mM HEPES buffer was found to be acceptable, a gas phase of 5% CO_2 in air, at least 25% adult bovine serum in the medium, avoiding storing for longer than a week at 4 °C, and subculture every one to three days.

A further modification of this method, the microaerophilus stationary phase culture (MASP), using small volumes in a stationary phase, produced rapid growth and high parasitaemias (Levy and Ristic, 1980) and has become the method most widely used

for *Babesia* cultivation. This method, using 60% medium-199 and 40% normal bovine serum with 15 mM HEPES and 100 mcg/ml streptomycin and 100 units/ml penicillin, depends on a critical depth of medium and the metabolism of the parasite to produce the reduced O_2 conditions required for optimal growth and re-invasion of the erythrocytes. Medium is changed daily and subcultures carried out at two- to three-day intervals. Cultures were initiated using defibrinated donor blood with a parasitaemia of 0.1–2%, to give a final PCV of 5–10%. The medium was titrated to pH 7.0 with 1N HCl and seeded into 25 cm^2 flasks or microtitre plates at a volume of 0.62 ml/cm^2. The column of medium above the parasitized cells acts as a barrier to O_2 exchange and its depth is crucial to successful cultivation. The settled cells become dark red to black due to the deoxygenation of the erythrocytes. This method requires a minimum seeding density, as the parasites modify the culture conditions, although there does not appear to be a period of adaptation to culture. Incubation was at 37–38 °C in 5% CO_2 in air. Subcultures were carried out after 48–72 hours with addition of fresh erythrocytes and reduction of the parasitaemia to 0.5–1% at each passage. Parasitaemias of 38% were obtained in this way. After five months in culture, babesiosis was induced in a splenectomized calf using parasites maintained in this way.

The importance of the gas phase was confirmed when it was shown that, by reducing the O_2 level to 2%, a wide range of fluid levels could be used, and cultures could be initiated with low percentage parasitized erythrocytes (PPE), below 0.1% (Rodriguez *et al.,* 1983). Using a gas phase of 5% CO_2, 2% O_2, 93% N_2, and by gradually reducing the PPE to initiate subcultures, Rodriguez *et al.* (1983) were able to clone *B. bovis* by limiting dilution. These culture conditions also allowed the isolation of parasites from carrier animals in which the parasites were undetectable on blood smears. Other effects on growth, as measured by PPE or by [^{3}H]-hypoxanthine incorporation, due to changes in culture conditions have also been reported, including serum supplements (Levy *et al.,* 1980) collection, preparation and storage of erythrocytes (Palmer *et al.,* 1982), pH and buffer of medium (Goff and Yunker, 1988) and the effect on varying initial PPE (Goff and Yunker, 1986). This has led to the continuous cultivation of several strains of *B. bovis* as well as other species of *Babesia* (Table 1). Microaerophilus stationary phase cultures are asynchronous, containing both trophozoite and paired merozoites at any one time. Under specific culture conditions, a threshold number of infected erythrocytes will be obtained, after which exponential growth ceases. However, at this level individual parasite development will continue, eventually resulting in a higher proportion of mature merozoite-infected erythrocytes (Goff and Yunker, 1986).

Babesia bovis-infected erythrocytes could be stored for up to 30 days at 4 °C (Erp *et al.,* 1980b) and *B. divergens* and *B. major* for up to eight weeks with a once weekly subculture (Konrad *et al.,* 1985), which would then resume growth when returned to 38 °C. The most efficient method for the cryopreservation of *B. bovis* was found to be the freezing of extracellular parasites in a solution of 10% wt/vol polyvinyl pyrrolidone (PVP –40) in Pucks saline G (PSG) containing 20 g glucose, using a cooling rate of 20 °C/min (Palmer *et al.,* 1982). Although cultures could be reestablished from thawed, infected erythrocytes, the plating efficiency was relatively low. A similar method has been used successfully for *B. bigemina* (Vega *et al.,* 1985b) except that VegayMartinez (VYM) solution replaced PSG, and a low O_2 gas phase

TABLE 1. Cultivation of *Babesia* species.

Host	Species	Culture	Reference
Bovine	*B. bovis*	short term	Erp *et al.,* 1978; Timms, 1980
		continuous	Erp *et al.,* 1980a; Levy and Ristic, 1980; Palmer *et al.,* 1982; Rodriguez *et al.,* 1983 Goff and Yunker, 1986, 1988
		bulk	Timms and Stewart 1989; Crowe, 1991
		tick stages	Droleskey *et al.,* 1983; Bhat *et al.,* 1979
	B. divergens	continuous	Vayrynen and Tuomi, 1982; Pudney, 1984; Donelly *et al.,* 1984; Konrad *et al.,* 1984, 1985; Gorenflot *et al.,* 1991
		bulk	Pudney, 1984
	B. bigemina	short term	Timms, 1980
		continuous	Vega *et al.,* 1985a; Montenegro-James *et al.,* 1987
	B. major	continuous	Donelly *et al.,* 1984; Konrad *et al.,* 1985
Equine	*B. equi*	lymphocytes	Schein *et al.,* 1981; Moltmann *et al.,* 1982; Zweygarth *et al.,* 1984; Rehbein *et al.,* 1982
	B. caballi	tick stages	Kurtii *et al.,* 1983
Canine	*B. canis*	short term	Levy and Ristic, 1980
Murine	*B. rodhaini*	short term	Jack and Ward, 1980; Barry, 1982a, 1982b
	B. microti	short term	Bautista and Kreier, 1979
Deer	*B. odocoilei*	continuous	Holman *et al.,* 1988

was necessary for resuscitation. *Babesia divergens* growing in human erythrocytes was cryopreserved using a standard *P. falciparum* method (Pudney, 1984), with 10% dimethyl sulphoxide (DMSO) in complete growth medium and plunging into liquid N_2; human erythrocytes are less susceptible to haemolysis on thawing than bovine red cells.

A modification of both the culture system and the cryopreservation technique for *B. bovis* permitted continuous growth of large volumes of this species *in vitro* and enabled cultures to be established directly from liquid N_2 stabilates (J.S. Crowe, personal communication). The stabilates contained infected erythrocytes frozen in medium-199 with Earles salts and 40% v/v fresh bovine serum buffered with 22.5 mM HEPES at a haematocrit of 20% with the addition of 2% DMSO. Freezing was performed at approximately 10 °C/min before storage in liquid N_2. Stabilates were thawed at 37 °C followed by one wash in 15 vols of medium-199 with 40% bovine serum, and centrifuged at 3,000 g for 10 min. The pellet was resuspended in 7 ml medium with 10% v/v erythrocytes in a 25 cm^2 flask. Flasks were gassed with 4% O_2, 5% CO_2, 91% N_2 and incubated lying flat on an orbital shaking incubator at 37 °C and 50 rpm. The doubling time of the parasites was approximately 24 hours and parasitaemias of 25% were routinely obtained. Medium was replaced at 24-hour intervals, 25 ml of medium was used in 75 cm^2 flasks for larger-scale cultures.

Babesia divergens, like *B. bovis,* is a small *Babesia* of bovids, which can also cause fatal disease in splenectomized humans and in intact gerbils. It can also be maintained in culture (Vayrynen and Tuomi, 1982). The ability to grow *B. divergens* in a small laboratory animal, together with *in vitro* culture of isolates from either cattle (Vayrynen and Tuomi, 1982; Konrad *et al.,* 1984; Gorenflot *et al.,* 1991), a human isolate maintained in gerbils, `J' strain (Pudney, 1984), or directly from human isolates (Gorenflot *et al.,* 1991), combined with equally good *in vitro* growth in either bovine or human erythrocytes (Pudney, 1984; Flory *et al.,* 1990), has proved particularly useful.

A more detailed review on *Babesia* vaccines is given elsewhere in these proceedings, so I will only briefly outline the type of work which has resulted from the establishment of continuous *Babesia* cultures. This can be divided into two areas, the use of soluble antigens secreted into culture media for vaccination, and the use of live, attenuated parasites or parasite antigens produced in culture as a source of parasite antigen for vaccination, or for the production of monoclonal antibodies for the further examination of specific antigens. Immunization of cattle with cell free soluble antigens prepared from suspension culture supernates (Erp *et al.,* 1978, 1980a) was shown to provide protection against a homologous tick-borne challenge exposure with virulent *B. bovis* organisms (Smith *et al.,* 1979, 1981). Similarly, soluble antigens from the MASP method (Levy and Ristic, 1980) are effective in inducing protective immunity to bovine babesiosis (Kuttler *et al.,* 1982). Immunochemical analysis of *B. bovis* supernates has demonstrated that at least three parasite exoantigens are released *in vitro,* and these have been characterized as proteins with Mr of 30–40 kDa (James, 1984). However, although trials conducted with *B. canis* and *B. bovis* have shown that soluble exoantigens found in culture supernates are highly immunogenic after homologous challenge (Molinare *et al.,* 1982; Kuttler *et al.* 1983); after heterologous challenge these antigens have only conferred partial protection (Timms *et al.,* 1983). The soluble antigens of cultured *B. canis* have also been characterized (Azzar *et al.,* 1990), and a vaccine produced from short-term *in vitro* culture by Merieux.

Exoantigens from *B. divergens in vitro* used in vaccination trials in gerbils demonstrated protection against virulent homologous and heterologous challenge (Gorenflot *et al.,* 1990). Immunoprecipitated soluble antigens from *B. divergens* of human and bovine origin showed similarities in the immunodominant polypeptides produced using serum from man, gerbil and ox, the three potential hosts of this species (Gorenflot *et al.,* 1991). A monoclonal antibody (MAb 13.4) (Flory *et al.,* 1990) raised against the Weybridge (bovine) strain of *B. divergens* has been shown to react with a 24-kDa antigen present on the surface of *B. bovis* and *B. bigemina* merozoites, as well as on *B. divergens* merozoites from bovine cultures. This MAb inhibits invasion *in vitro* and the affinity purified antigen is partially protective against subsequent challenge with *B. divergens.* MAb 13.4 will bind to `J' and Weybridge merozoites which have been cultured in bovine erythrocytes, but not after their culture in human erythrocytes, showing potentially different erythrocyte receptors for invasion. Both strains can cause fatal infections in gerbils and return to bovine culture, showing transient antigenic alterations (Flory *et al.,* 1990). A high degree of antigenic cross-reactivity between *P. falciparum* and *B. bovis* parasites and the sharing of common antigenic epitopes has also been observed using monoclonal antibodies

produced against individual culture-derived parasite polypeptides. Animals vaccinated with a merozoite vaccine prepared from spinner flask cultures of *B. bovis* were protected from death following challenge (Smith *et al.,* 1979). Both a *B. bovis* clone, selected on its rapid growth rate *in vitro,* and an attenuated strain produced *in vitro* by continuous propagation in bovine erythrocytes in cultures in which bovine serum was gradually replaced by equine serum have been successful in immunizing splenectomized calves (Yunker *et al.,* 1987), yearling heifers and two-year-old steers (Kuttler *et al.,* 1988), indicating the potential use of these culture-derived parasites as live immunogens. Using a different approach, the vaccine strain of *B. bovis,* Ka, could be maintained *in vitro* using MASP cultures for 174 days without a change in virulence. A combination of MASP cultures and a short period of batch culture or flow through cultures were able to produce titre quantities of heavily parasitized erythrocytes for vaccine use (Timms and Stewart, 1989).

Current knowledge of the behaviour of *Babesia* within the tick is incomplete. *In vitro* systems offer a means to study some of these host cell-parasite interactions under controlled conditions. Hoffmann (1972) studied the cyclical multiplication of *B. bigemina* in cultures of infected *Boophilus annulatus* organs. Various stages of the parasite were seen for 5–12 days, but there was no conclusive evidence that the parasites multiplied. There was no mention of whether these parasites were capable of invading organs from uninfected ticks. To investigate the pathological effect caused by *B. cabalii* in the vector tick, *Anocentor nitens,* cell lines were initiated from transovarially infected embryos. In culture the parasites were detected by light microscopy for three to five days but did not transform into other tick-associated stages, and were not seen after one week. There was no evidence of replication, and only limited development was seen in both primary cultures and young cell lines isolated from embryos of various tick species, including the vector (Kurtii *et al.,* 1983). Ronald and Cruz (1981) obtained similar results when they infected two *Boophilus microplus* cell lines with *B. bovis* kinetes from haemolymph and eggs. Attempts to infect cultured tick cells with the erythrocytic stages of *B. bovis* have also not resulted in the development of tick-associated stages of *Babesia* (Bhat *et al.,* 1979), although electron microscopic examination was able to show that when *B. microplus* cell cultures were inoculated with high concentrations of erythrocytic stages of *B. bovis,* the erythrocytic stage of the parasite apparently developed to a stage with many of the ultrastructural characteristics of the sexual stages of *B. bigemina* and *B. canis;* indicating that these parasites may have represented the sexual forms (Droleskey *et al.,* 1983).

The complete vertebrate life cycle of *B. equi* has been demonstrated *in vitro,* and the corresponding stages identified in the horse (Schein *et al.,* 1981). The exoerythrocytic stages observed were similar to those found in *Theileria* species with typical schizont stages. Equine lymphoblastoid cell lines were also infected and transformed by *B. equi* sporozoites as described for *T. annulata* and *T. parva* cell lines (Rehbein *et al.,* 1982). The first schizonts were detected on days 2–5, and, on days 8–10 after inoculation of the sporozoites, schizonts began to undergo merogony. The merozoites thus formed were able to infect horse erythrocytes *in vitro* and *in vivo.* The lymphoblastoid cells were subcultured after 10–14 days, and, on Giemsa staining, the percentage of infected cells reached 90–95%. The mode of replication of the parasitized lymphoid cells appears to be identical to the process in *T. parva* and *T. annulata*

cell cultures, with the division of the schizonts and their host cells being interdependent. After a period of five months (20 passages), the cell culture of *B. equi* contained only schizonts. Sporozoites did not invade horse erythrocytes in culture. Cultured schizonts, inoculated into a clean donor, induced piroplasms in the peripheral erythrocytes two days later. The ultrastructure of the exoerythrocytic stages of *B. equi* also shows striking similarities to the schizogony stages of the *Theileria* life cycle (Moltmann *et al.,* 1983).

The presence of exoerythrocytic schizonts, with possibly different drug sensitivities, could be an explanation for the fact that *B. equi* is difficult to treat with babesicidal drugs. The *B. equi*-infected continuous lymphoblastoid cell line BE10 (Rehbein *et al.,* 1982) was used to test the efficacy of halofuginone, parvaquone (Clexon) and oxytetracycline, all of which have been shown to have a schizonticidal effect in the treatment of bovine theileriosis, and the babesicidal drug diminazene aceturate which, although effective against *B. caballi,* is ineffective against *B. equi.* Parasite growth was determined by measuring DNA synthesis in drug-treated and control cells. Cells were incubated in the presence of drug for 72 hours in microtitre plates at 37 °C in 5% CO_2 in air, and then pulsed for six hours with [^{3}H]-thymidine. Both halofuginone and parvaquone caused over 80% inhibition of [^{3}H]-thymidine incorporation at either 0.02 or 5.0 ug/ml respectively, indicating that both compounds act on the schizont stages. Inhibition of over 60% was observed at a level of 50 ug/ml oxytetracycline, and only 40% for diminazene aceturate, indicating a far greater response to the schizonticidal drugs than the babesicidal drug, diminazene, and thus the possible potential for its use in the treatment of *B. equi* (Zweygarth *et al.,* 1984).

Other large-scale systems for *B. bovis* erythrocytic stages, apart from the spinner culture of Erp *et al.* (1980b) and the batch culture of Timms and Stewart (1989) include the use of rotated 75 cm^2 flasks (J.S. Crowe, personal communication). *Babesia divergens* was also grown in batch cultures by Pudney (1984) using the human `J' strain grown with human erythrocytes in 200 ml volumes in a Techne microcarrier stirrer. Greater than 20% parasitaemias were also routinely produced by the use of 50 ml volumes in 100 ml Erlenmeyer flasks on an orbital shaker, using the same method as for small cultures, i.e. 10% haematocrit in a gas phase of 5% CO_2, 2% O_2 and 93% N_2, with daily medium changes of RPMI 1640, with added glucose, hypoxanthine and 10% human serum and subcultured every three to four days. Up to 4×10^{12} parasites (Erp *et al.,* 1980b) were produced weekly and used for the isolation and study of mitochondria (M. Fry, personal communication).

Isolation of intracellular parasites, whilst retaining both parasite integrity and viability, is a constant source of concern for parasite biochemists and others requiring pure, viable material. However, the observation by Levy and Ristic (1980) that large numbers of merozoites of *B. bovis* accumulate in the medium following CO_2 deprivation has provided a simple method for producing free parasites. In the case of *B. bovis* the parasites can be further concentrated using differential lysis. Winger *et al.* (1987) prepared isolated *B. divergens* merozoites by incubating cultures in a desiccator over soda lime for six to eight hours at 38 °C. Merozoites were further concentrated by a modification of the method of Levy and Ristic (1980) and the material containing merozoites and red cell ghosts was used for the production of monoclonal antibodies. Inhibition of merozoite invasion by these antibodies was later shown using the merozoite neutralization assay. Large

numbers of isolated *B. divergens* parasites were obtained for studies on O_2 uptake, by leaving cultures open to air for 24 hours and removing any remaining infected or uninfected red cells by centrifugation (M. Pudney and M. Fry, unpublished data). Centrifugation in a Percoll gradient has also been used to completely separate parasites *(B. bovis)* from red blood cells and stroma (Rodriguez *et al.,* 1986) as well as to concentrate infected erythrocytes. Isolation of *B. bigemina* from erythrocytes prior to concentration and purification was obtained by the use of glycerol-enhanced osmotic shock (Figueroa *et al.,* 1990).

Erythrocytes and intraerythrocytic *Babesia* parasites, like *Plasmodium,* are unable to synthesize purine nucleotides *de novo* and therefore rely on obtaining these metabolites from the extracellular medium (Irvin and Young, 1979; Conrad, 1986). In accordance with this obligate requirement, nucleoside transport has been found to be induced in bovine erythrocytes upon infection with *B. bovis* (Gero, 1989). Bovine erythrocytes, which are virtually impermeable to glucose, were also shown to display a dramatic increase in glucose permeability following infection with *B. bovis* (Upston and Gero, 1990). Evidence has also been obtained for the insertion of a parasite specific nucleoside/nucleobase transporter into the membranes of the bovine (host) red cell. Thus, whereas normal (non-parasitized) bovine red cells are essentially incapable of transporting nucleosides across their membranes, the invasion of these cells by *B. bovis* introduces a transporter that can be inhibited by classic nucleoside transport inhibitors (Matias *et al.,* 1990).

The continuous cultivation of *P. falciparum* and the $[^3H]$-hypoxanthine assay described by Desjardins *et al.* (1979) has become a standard tool in antimalarial research, with widespread applications and modifications. This system has now been applied to both *B. divergens* (Pudney and Matthews, 1985) and *B. bovis* (Nott *et al.,* 1990) with growth in continuous culture. Using a human isolate of *B. divergens* maintained by serial passage in gerbils, cultures have been initiated and maintained continuously in parallel strains in bovine and human erythrocytes (Pudney, 1984). Culture material readily infects splenectomized calves and intact gerbils, and parasites from the bovine strain grow well in human erythrocytes and vice versa. Tests were carried out using microtitre plates in a total volume of 150 ul/well and an initial parasitaemia of 2%. Trays were incubated at 37 °C for 24 hours in a humidified chamber in the *Babesia* gas mixture, following which $[^3H]$-hypoxanthine was added and the trays incubated for a further 24 hours. Cells were harvested using a cell harvester which lyses the cells and deposits washed particulate material onto filter papers. These discs, placed individually into scintillation vials, are counted to calculate the $[^3H]$-hypoxanthine incorporated (Pudney and Matthews, 1985). Variations on this assay described by Nott *et al.* (1990) included the use of sterile water in the peripheral wells to ensure adequate humidity, a total volume of 200 ul/well and an initial parasitaemia of 1%, and pulsing with hypoxanthine for 18 hours instead of 24 hours. Large numbers of compounds can be screened for activity with this system and then IC_{50} levels obtained for active drugs for comparison with other compounds.

Incorporation of $[^3H]$-hypoxanthine was found to be a reliable indicator of parasite growth, closely paralleling the increase in percentage of parasitized cells. The activity of the standard antibabesial compounds for *B. divergens* gave the following IC_{50}s: quinuronium sulphate, $3.4 \times 10^{-8}M$; ethidium bromide $4.7 \times$

10^{-8}M; imidocarb dipropionate 1×10^{-7}M; pentamidine isethionate 3.6×10^{-7}M; diminazene aceturate 3.7×10^{-7}M and amicarbalide isethionate 4.6×10^{-7}M. Comparable results for *B. bovis* were 1.27×10^{-7}M; 9×10^{-7}M; 5.8×10^{-7}M; 5.8×10^{-7}M; not done, and 1.25×10^{-6}M, respectively. There is considerable interspecies variation in drug response within *Babesia,* and although *B. divergens* was used in a primary screen as a representative of the small *Babesia* spp., all active compounds were then screened against *B. bovis* (Pudney, 1984). The level of activity is similar, although consistently lower in *B. bovis.* This may in part be due to the test system which has been optimized for *B. divergens.* Further confirmation of the usefulness of this test system was the indication of the activity of a series of hydroxynaphthoquinones (HNQ) which had been inactive in the *B. rodhaini* model. The most active of these HNQs, 720C (buparvaquone) was subsequently shown to have activity against *B. divergens* in both gerbils and cattle (unpublished data). Another advantage of this system is the unique opportunity to investigate drug effects in different host cells. In the majority of cases the compounds were less effective in the human cells. Evaluation of some standard antimalarial drugs showed antibabesial activity, which was however very low compared to their corresponding antimalarial activity; it can be seen why such drugs are ineffective against human cases of babesiosis. One of the HNQs shown to have antibabesial activity in both human and bovine erythrocytes, 566C, is currently in clinical trials for treatment of malaria, and in *Pneumocystis carinii* and *Toxoplasma gondii* in AIDS patients; the *in vitro* assay would indicate that this compound would be suitable for the treatment of human cases of babesiosis.

This assay has also been used as a more general research tool. The HNQ series of drugs are known to act on the electron transport chain of the mitochondria. *In vitro* effects of standard electron transport inhibitors has indicated the presence of a functional, mitochondrial electron transport pathway in *B. divergens.* Approximately 50% of the O_2 uptake by intact cells is inhibited by classical electron transport inhibitors. Cytochemical staining has also indicated the presence of cytochrome oxidase activity contained within a large, cristate and probably single mitochondrion per parasite (M. Fry, unpublished information). Nott *et al.* (1990) found imidocarb and amicarbalide to be active against *B. bovis* in the 10–40 nM range. They also demonstrated the activity of tubercidin and other nucleoside analogues, as well as the antibiotic tetracycline, the HNQ menoctone, and pyrazofurin, an inhibitor of pyrimidine biosynthesis. Both phloretin and phlorizin inhibited babesial growth indicating that active transport rather than simple diffusion mechanisms operate across the membrane of the parasitized host cell or that of the intracellular parasite. Also shown was the activity of a series of antimalarial diMannich bases. Such results illustrated the usefulness of this *in vitro* assay system, both for assessing inhibitors of a specific biochemical function, and for testing compounds of known activity and novel structures. It is difficult to determine the reason for the difference in efficacy of the antibabesial standards as reported by Pudney and Matthews (1985) and Nott *et al.* (1990), but may be due to the difference in species and/or strain of species used, or to the technical differences in the assay, which would be expected to produce minor differences. However, the efficacy of the compounds in two independent laboratories has been confirmed.

Cultivation of *Babesia,* especially of bovine origin, has now developed to the stage where several species and strains are culture adapted and can be reliably maintained in continuous culture and stored in liquid N_2. Unfortunately the necessity for daily medium changes has not yet been overcome, although some strains can be left over a weekend period without attention. Automation of this process would greatly increase the usability of the system. The *in vitro* cultures have lived up to their potential in several areas and have been used to make significant advances, particularly in the area of soluble exoantigens for vaccine use, the traditional use of producing attenuated parasites, the provision of antigens for the production of monoclonal antibodies, for investigations on immunological response, on parasite entry into the red cell, and certain aspects of host specificity. The culture method has enabled biochemical investigations to be carried out on viable material, even if over short time periods. The determination of the exoerythrocytic stage of *B. equi* was confirmed by the use of the culture system which has clearly shown this aspect of the developmental cycle. This has brought into doubt the classification of this species as a true *Babesia,* but it has also highlighted possible reasons for the lack of success in the treatment of *B. equi.*

As yet, little success has been achieved in comparable cultures of the developmental stages in the tick. The wider use of the [^{3}H]-hypoxanthine uptake assay for the identification of new drugs, and the investigation of their site(s) of action will build on the success already achieved in this area. The establishment of drug efficacy in the treatment of babesiosis is complicated by the wide variety of hosts and *Babesia* species which have differing degrees of drug tolerance and susceptibility. The development of culture systems for other species of *Babesia* would assist in characterizing the biochemical, as well as antigenic differences, that exist between them.

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Progress with the cultivation of *Cowdria ruminantium* in endothelial cells

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The *in vitro* cultivation of *Cowdria ruminantium* remains a fragile system despite considerable progress made since its initial development. A minimum of 13 stocks of *C. ruminantium* have been cultivated successfully *in vitro* in endothelial cells. Organisms are cultured on bovine or ovine endothelial cells in the Glasgow modification of Eagles minimal essential medium (Gibco: GMEM) or Leibovitz L-15, both with additives. Cultured organisms have been used as antigen in immunofluorescent tests, in biochemical studies in which immuno-dominant proteins were isolated, for the isolation of *Cowdria* DNA and in studies on the development of a tissue culture inoculum against heartwater. The morphology and life-cycle of the organism have been studied and attenuation of the Senegal stock of *C. ruminantium* has been reported.

INTRODUCTION

The successful *in vitro* cultivation of *Cowdria ruminantium,* which took place relatively recently, has stimulated renewed interest in the disease (Bezuidenhout, 1987, 1988). *In vitro*-cultured organisms or infected cells have been used as antigen in serological tests such as in the direct and indirect fluorescent antibody test (Bezuidenhout *et al.,* 1985; Yunker *et al.,* 1988; Martinez *et al.,* 1990) in an enzyme-linked immunosorbent assay (ELISA) (Viljoen, 1985) and during the development of monoclonal antibodies used in the competitive ELISA (Jongejan *et al.,* 1990a). Furthermore, infected tissue cultures have been used for the isolation of *Cowdria* DNA in studies aimed at establishing DNA libraries and DNA probes (Ambrosio *et al.,* 1987; Wilkins and Ambrosio, 1989; Jongejan, 1990; Yunker, 1991). Biochemical studies have also been conducted on the organisms in culture and immuno-dominant proteins isolated (Jongejan and Tielemans, 1989; Rossouw *et al.,* 1990). At the Veterinary Research Institute (VRI), Onderstepoort, a study has also been made into the possible replacement of the present blood vaccine with a live tissue culture inoculum. During studies on the *in vitro* cultivation of *C. ruminantium,* the Senegal stock was attenuated through continuous cultivation in endothelial cells (Jongejan, 1991).

CELL LINES SUITABLE FOR *IN VITRO* CULTIVATION OF *COWDRIA*

The first successful *in vitro* cultivation of the organism was in bovine umbilical cord endothelial cells (E5) (Bezuidenhout *et al.,* 1985). The cells were isolated according

to the method of Maruyama (1963) (B.J. Erasmus, personal communication). Subsequently, Yunker *et al.* (1988) tested 11 cell lines, other than E5, for their susceptibility to *Cowdria.* They found that three of these cell lines, all established from bovine arterial endothelium according to a modification of the method described by Hirumi and Hirumi (1984), supported the growth of the organisms on a continual basis, while a fourth, established from foetal bovine heart endothelium (American type culture collection CRL-1395), supported the multiplication of the organism in some instances. Bovine umbilical endothelial cells (BUE), isolated according to a modification of the method of van de Wiel *et al.* (1989), were used by the group in Utrecht during their studies on the organism in culture (Jongejan *et al.,* 1990b; Jongejan, 1990). An ovine endothelial cell line (SBE189) was recently established at the VRI, Onderstepoort, according to a modification of the method described by Phillips *et al.* (1979) (S. Brett, unpublished data). This cell has supported the growth of *Cowdria* since its fourth passage and is presently at passage 50. Another ovine cell, isolated from pulmonary artery endothelium, was found to be susceptible to *Cowdria* (Byrom *et al.,* 1991).

CULTURE MEDIA

The Glasgow modification of Eagle's minimum essential medium (GMEM, Gibco version containing L-Glutamine 292 ug/ml), locally produced at the VRI, Onderstepoort, with the addition of tryptose phosphate broth (TPB), bovine serum and antibiotics, was used during the first successful *Cowdria* cultivation experiments. This medium is still in use with excellent results in the yielding of highly infected *Cowdria* cultures (Bezuidenhout *et al.,* 1985; Bezuidenhout, 1987; S. Brett, unpublished results). The GMEM (commercially available), supplemented with TPB, L-Glutamine, foetal calf serum (FCS) at 10% (sometimes even as low as 3%) and antibiotics, is used widely (Yunker *et al.,* 1988; Jongejan *et al.,* 1990b; Martinez *et al.,* 1990). Byrom and Yunker (1990) obtained better results by using Leibovitz L-15 medium supplemented with 0.45% glucose, 5% foetal bovine serum, 292 ug/ml L-glutamine and antibiotics. The pH of the GMEM culture medium is adjusted to 6.0–6.5 by gassing with CO_2 and the L-15 by adding HCL.

CULTURE METHODS AND CONDITIONS

Irradiation and cyclohexamide were used in early culture studies to inhibit the growth of cells in order to facilitate initial infection with *Cowdria* (Bezuidenhout *et al.,* 1985; Yunker *et al.,* 1988). It is now generally accepted that the irradiation of cells is not a prerequisite for successful initial infection of cultures (Bezuidenhout, 1987, 1988; Byrom and Yunker, 1990; Jongejan *et al.,* 1990b). Very little or no success has been reported after treatment of cultures with cyclohexamide (Bezuidenhout, 1987; Yunker *et al.,* 1988). A very simple and practical method to facilitate closer contact between the cells and inoculum, now widely incorporated in the cultivation of *Cowdria,* is to place the cultures on a slow-rocking platform (2–4 rocks/min), or, for mass production, to use a roller bottle system (S. Brett, unpublished results). Cultures are

incubated at 37 °C. No conclusive information has been published on the effect of temperature on the growth of *Cowdria* in cultures.

The storage of *Cowdria* at low temperatures remains a problem due to the loss of infectivity during the freezing/thawing process. However, it was found that the freezing of organisms in liquid nitrogen while still intra-cellular as small colonies (two days after infection) gives excellent results when such cultures are revived (S. Brett, unpublished results).

INOCULATION OF CULTURES

Sheep or goat blood collected in heparin (50 units/ml) with or without sucrose phosphate buffer during the febrile reaction is mostly used for the inoculation of cultures (Bezuidenhout *et al.,* 1985; Yunker *et al.,* 1988; Jongejan *et al.,* 1990b; Martinez *et al.,* 1990). It appears that fresh blood gives better results than frozen blood. A variety of blood components such as leucocytes, neutrophils and macro-phages have been used as inocula without success (Byrom and Yunker, 1990; Byrom *et al.,* 1991) but blood plasma gives better results.

In the case of mice infected with murinotropic stocks, pooled samples of infected organs such as spleen, liver, heart, kidney and blood have been used successfully as inocula and, more recently, the lungs alone have been used (S. Brett, unpublished results). Inocula of tick stabilates prepared from *C. ruminantium*-infected ticks have also been successfully cultivated *in vitro* (Bezuidenhout *et al.,* 1985; Bezuidenhout, 1987). Due to their toxicity to the cultured cells and the fact that they often contain bacterial contamination, tick stabilates are, however, not regarded as a very effective inoculum.

STOCKS OF *COWDRIA RUMINANTIUM* CULTURED

The successful *in vitro* cultivation of a great number of *Cowdria* stocks have been reported (Table 1). The Kumm stock appears to be the only one to yield constantly negative results (Bezuidenhout, 1987) even on the ovine endothelial cell line (SBE189) which gives excellent support to other stocks (S. Brett, unpublished data). At this stage it would be unwise to lay too much emphasis on any differences reported between the growth potential of different stocks, especially as they were not always subjected to the same culture conditions.

OBSERVATIONS OF *COWDRIA* IN CULTURE

After *C. ruminantium* is taken up by endothelial cells in culture, it divides by binary fission within intracytoplasmic vacuoles, forming colonies of reticulate bodies. After three to four days in culture, the reticulate bodies develop into smaller intermediate bodies characterized by an electron-dense core. The intermediate bodies condense further into electron-dense elementary bodies which rupture from the cells and are released into the culture medium. From here they invade other endothelial cells thus

TABLE 1. Stocks of *Cowdria ruminantium* cultivated in endothelial cells.

Stock[*]	Cell line[†]	Reference
Ball 3	E5	Bezuidenhout *et al.,* 1985; Bezuidenhout, 1987; Byrom and Yunker, 1990
Crystal Springs	BA886; BA987; FBHE	Yunker *et al.,* 1988; Byrom and Yunker, 1990
Gardel	E5	Martinez *et al.,* 1990
Highway	BA987; BPA689; OPA	Byrom and Yunker, 1990 Byrom *et al.,* 1991
Kwanyanga	E5	Bezuidenhout, 1988
	SBE189	Brett, unpublished data
Lemco T3	BA987; BPA689	Byrom and Yunker, 1990
Mali	E5	Bezuidenhout, 1988
Mbizi	BVE	Byrom *et al.,* 1991
Nonile	E5	Bezuidenhout, 1988
Nyatsanga	BVE	Byrom *et al.,* 1991
Palm River	BA987	Yunker *et al.,* 1988
Senegal	BUE	Jongejan *et al.,* 1990b
	E5	Martinez *et al.,* 1990
Welgevonden	E5	Bezuidenhout, 1987 Martinez *et al.,* 1990
	SBE189	Brett, unpublished data
	BA886	Yunker *et al.,* 1988; Bezuidenhout, 1988
	BUE	Jongejan, 1990
	BA897; FBHE; BPA987	Yunker *et al.,* 1988; Byrom and Yunker, 1990

[*]Two additional stocks viz. Nigeria and Zwimba have also been cultivated *in vitro* in Harare, Zimbabwe (C.E. Yunker, personal communication).
[†]All the cells are of bovine origin except the SBE189 and OPA which are from ovine origin.

initiating a new infectious cycle which lasts between four and six days (Jongejan *et al.,* 1990b). Earlier reports of the detection of *Cowdria* colonies only 11–14 days after inoculation (Bezuidenhout, 1987; Yunker *et al.,* 1988) were probably due to a low initial infection rate allowing detection only after the organisms had multiplied through one or more cycles. After gaining more experience and information on the *in vitro* cultivation of *Cowdria,* colonies are now detected as early as two days after inoculation (S. Brett, unpublished observation; Byrom *et al.,* 1990; Jongejan *et al.,* 1990b).

Depending on the level of infection of cultures, a cytopathic effect (CPE) is observed usually from day 5 after infection, but sometimes only after 11–14 days or

even later. Cytopathic effect is especially noticeable if the cultures are kept in constant movement as on a rocker or in roller bottles. Under such conditions CPE is seen as clear streaks in the monolayer where affected cells are in the process of detaching or already detached (S. Brett, unpublished observation; Yunker *et al.,* 1988; Byrom *et al.,* 1991).

DISCUSSION

Apart from the usual hazards associated with culture work, such as sporadic contamination of cultures and poor performance of cells or inocula in culture, techniques for the *in vitro* cultivation of *Cowdria* are now fairly standardized and reasonable to excellent yields are obtained. It is possible to produce the organism in bulk in roller bottles, but only with great care, which includes monitoring of cultures in order to ensure correct timing of the optimal release of elementary bodies.

Most bovine endothelial cells presently in use for the cultivation of *Cowdria* seem to be suitable for this purpose provided they are kept healthy. They grow well under most laboratory conditions but their division rate is rather slow compared to most other cells used for vaccine production. The ovine endothelial cell line (SBE189) recently established at the VRI, Onderstepoort, supports the growth of *Cowdria* very well and is, in most cases, more suited to a high rate of initial infection than the E5 cell line. However, as in the case of the E5 cell line, it also does not support the growth of the Kumm stock, for which a murine endothelial cell line might have to be developed. There does not seem to be an urgent need to identify any other cell for the cultivation of *Cowdria,* unless such a cell could facilitate the process of attenuation.

Some of the objectives identified earlier with regard to the *in vitro* cultivation of *Cowdria* have been achieved. These include aspects such as the use of cultured organisms as antigen in serological tests and biochemical and DNA studies. Infected cell cultures were also studied in detail to clarify the life cycle of *Cowdria* and the principle of attenuation of organisms in culture has also been established (Jongejan *et al.,* 1990b; Jongejan, 1991). Perhaps the most important objective is the use of cell cultures for the development of a safe and effective vaccine against heartwater. The main reasons for this are the lack of cross-protection between the different stocks, the labile nature of the organism, the inability of inactivated organisms to stimulate a protective immunity (S. Brett and J.D. Bezuidenhout, unpublished data) and the impracticality of using the intravenous route for effective inoculation. Of course it is realized that with the latest techniques in molecular biology, it is, at least in theory, possible to overcome most of these problems. If protective proteins can be identified and the genes coding for them incorporated into vectors that will stimulate cellular immunity, together with the latest adjuvants, it could turn out to be a very effective and safe vaccine. However, judging from the slow progress made with research on other, similar or even more simple organisms, this could take a long time.

It is, therefore, important that, if we wish to exploit the developments made so far towards improving practical measures of disease control, aspects such as stability of the organism in infected culture material and the potential of blood as an infective inoculum should receive more attention. It has been established that enough organisms can be cultured in a roller bottle (800 cm^2) to vaccinate 20,000 sheep (S. Brett,

unpublished data). However, more studies need to be done on preserving the final product in order to maintain a high level of infectivity after freezing and thawing. Freeze-drying could play an important role in this regard (du Plessis *et al.,* 1990).

Attenuation of organisms *in vitro,* as already established for the Senegal stock (Jongejan, 1991), should also be studied in other stocks. If attenuated stocks are stable and the protection that they offer against other important stocks reliable, they would hold great promise in the development of future live vaccines. Other aspects also identified as objectives (Bigalke, 1987) that still need attention are the cloning of endothelial cells and, perhaps even more urgently, the cloning of the different stocks of *C. ruminantium.* The development of a plaque assay for this purpose and also for the quantification of infective material is, therefore, of utmost importance.

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In vitro cultivation of *Anaplasma marginale*

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INTRODUCTION AND HISTORICAL PERSPECTIVE

An *in vitro* culture system for *Anaplasma marginale* is an important objective of anaplasmosis research. The logistics and expense of working with cattle places constraints on many aspects of research on this disease, particularly in areas like chemotherapeutic and vaccine trials. Although there is no continuous culture system presently in use, previous attempts have provided valuable information on the behaviour of the organism and suggest that a continuous culture system is possible. As research into the biology of *Anaplasma* continues, it is becoming clear that this organism is unusually complex among rickettsiae. It is important for anyone working in the area of *in vitro* propagation to understand the historical progression of culture attempts to appreciate the fastidious nature of this organism outside of its natural environment.

The first reported attempt at culturing *A. marginale* was in 1915 (Veglia, 1915) in South Africa. There have been numerous attempts since then, primarily involving propagation of infected erythrocytes in various liquid medium preparations. Many attempts have gone unreported. Following the techniques of Trager and Jensen (1976) for cultivation of the erythrocytic stages of *Plasmodium,* a number of short-term culture systems for *A. marginale* have been reported (Davis *et al.,* 1978; Kessler *et al.,* 1979; Kessler and Ristic, 1979; Mazzola and Kuttler, 1980; McHolland and Trueblood, 1981). More elaborate systems such as continuous flow culture have also been attempted (Erp and Fahrey, 1975). Most of these systems have resulted in increased parasitaemia over one to three days followed by a gradual decrease or stasis. While the initial increase in parasitaemia observed may be attributed in part to development of mature inclusion bodies from initial bodies, it has been demonstrated that infection of uninfected bovine erythrocytes by stages in ovine erythrocytes did occur during the first 48 hours in culture (Kessler and Ristic, 1979). Viability of *A. marginale,* as determined by animal inoculation, has been maintained for 33 days (Kessler *et al.,* 1979). Parasites are also capable of maintaining protein and DNA synthesis by incorporating precursors for up to five days in culture although replication appears to cease after one or two days (Davis *et al.,* 1978). Short-term culture systems have been useful in studying aspects of the basic biology of the organism in an artificial system and may eventually be applied to successful long-term systems.

There have been several attempts to cultivate *Anaplasma* in nucleated cells. The most interesting report is by Marble and Hanks (1972), who inoculated cultured rabbit

bone marrow cells with bovine erythrocytes infected with *A. marginale.* Cultures were passaged weekly and animals were periodically inoculated with culture material. Their results indicated that cultures remained infective to cattle for 140 days. Organisms could not, however, be identified in cultures using Romanowsky or acridine orange staining techniques. The results were encouraging but they have not been duplicated. Swann *et al.* (1981) reported that, in a similar attempt, increasing numbers of organisms could be identified in rabbit bone marrow cells using immunofluorescence for 6–12 days but began declining after 20 days. Hidalgo (1973) reviewed several attempts to infect bovine bone marrow cells with the erythrocytic stage of *A. marginale* but none were successful. In an eight-year progress report, McHolland and Trueblood (1981) detailed attempts to infect bovine marrow, lymph node, embryonic kidney, turbinate and spleen cells, and mouse fibroblasts and chick embryo fibroblasts with the erythrocytic stage. They reported that all these cells took in organisms and survived for varying periods of time but no replication was observed. Infection of lymph node organ cultures with stored, infected erythrocytes has also been attempted (Trueblood and Bear, 1973). Organisms were identified in smears of lymphoid cells with fluorescent antibody and Giemsa staining techniques but replication was not apparent. Supernatant from these cultures was, however, infective for cattle up to 40 days after initiation.

Attempts to infect vertebrate nucleated cells with the erythrocytic stage of *A. marginale* have thus far produced inconclusive results. This stage is infective when mechanically transmitted between cattle but there is no evidence that the organism replicates in cells other than erythrocytes *in vivo*. While it has been known for many years that *A. marginale* can be transmitted mechanically or biologically by blood feeding arthropods (Dikmans, 1950), it has now been demonstrated that this organism can undergo a complex development within different cells of ixodid ticks (Kocan *et al.,* 1980, 1988, 1990a, 1991; Stiller *et al.,* 1989). The infective form for the tick cells is likely to be the erythrocytic stage. The first attempts to infect arthropod cell lines involved inoculation onto cells derived from *Aedes albopictus* (Mazzola *et al.,* 1976, 1979). These cells were shown to phagocytize infected erythrocytes and organisms were observed intracellularly for up to 60 days but without apparent replication. Infectivity was maintained for 21 days as determined by calf inoculation. Samish *et al.* (1988) inoculated a cell line derived from *Dermacentor variabilis* embryos with infected erythrocytes. It was reported that phagocytized *Anaplasma*-like particles, identified with fluorescent microscopy, increased in number over 20 days and two passages, but no corroborating evidence of replication was provided. Hidalgo *et al.* (1989a) used a tick gut stage of *A. marginale* as inoculum for a cell line derived from embryonic *D. variabilis.* Using a fluorescent monoclonal antibody to the erythrocytic stage, the authors reported a generalized fluorescence in infected cultures that was not found in control cultures inoculated with uninfected tick material. Electron microscopy revealed intracellular inclusions which appeared to contain rickettsial-like organisms. Cultures were maintained for 22 passages over 11 months. Inoculation of culture material into susceptible animals did not induce infection and animals were susceptible to challenge although some pre-challenge sera did react at a 1:10 dilution in an ELISA test (Hidalgo *et al.,* 1989b).

In examining the relatively short list of reports on attempts to cultivate *A. marginale* there appears to be a consistent theme which might serve to encourage further

work. In both erythrocytic and a number of nucleated cell cultures the organism appears to survive intercellularly for an extended period of time. Replication has not been demonstrated but survival after extended passages might allude to this. The potential benefits of a successful continuous culture system would warrant its further investigation.

CURRENT RESEARCH

In light of previous unequivocal findings and the continued need for an *in vitro* system to study *A. marginale,* research was recently initiated at the Oklahoma State University Anaplasmosis Research Laboratory to further investigate possible culture systems. Initial efforts have focused on uses of short-term erythrocytic cultures and measures to increase the longevity of these culture systems. Efforts are currently under way to evaluate potential continuous culture systems using tick-derived stages on *A. marginale.*

Short-term erythrocyte cultures

The first culture attempts of *A. marginale* by our group were based on the Trager and Jensen (1976) model for the cultivation of *Plasmodium* with different variables of this system being manipulated. Initiation of cultures with blood at different levels of parasitaemia were attempted. Cultures initiated with low level parasitaemic blood (1% or less) resulted in no noticeable increase. Cultures initiated with infected blood during log phase growth generally resulted in varying degrees of increase over a 48-hour period. Cultures initiated with 10–20% infected erythrocytes increased approximately 5% above the initial parasitaemia but those initiated with 50% or more infected erythrocytes increased by 30–40%. After six to seven days the number of infected erythrocytes decreased in all cultures. The following variables were also investigated in our short-term cultures: adjustment of pH between 7.0 and 7.4, varying serum content between 10% and 40%, using calf serum instead of foetal calf serum, increasing incubator temperature to 40 °C, increasing CO_2 levels from 5 to 10%, varying the size of incubation flasks and increasing glucose levels in medium from 2 to 4 g/L and glutamic acid levels from 20 mg/L to 40 mg/L. We have also attempted to use a modification of a suspension culture method for *Babesia bovis* (Erp *et al.,* 1978) in which cultures were kept in suspension using a cell rocker or rotator. None of the modifications employed here resulted in a significant departure from previous findings.

Cultures were initiated based on the microaerophilus stationary phase (MASP) system of Levy and Ristic (1983) for the cultivation of *B. bovis.* In this system the depth of the column of medium above stationary cells was critical in maintaining the low oxygen environment necessary for continued development of *Babesia.* It was also found that parasite multiplication did not persist beyond three to four days after subculturing and reducing parasitaemias to 0.5–1% resulted in maximum growth. In using this system with *A. marginale*-infected erythrocytes, we seeded cultures at parasitaemias of 1, 5 and 10% and subcultured every two days at a ratio

of 1:3. This resulted in a rapid dilution of parasitaemia over several subcultures. Cultures were next seeded at 5% and subcultured at a 1:1 ration every four to five days. Inclusion bodies could still be identified after five subcultures but at a very low level. These trials are continuing to determine if a persistent low level infection can be maintained.

Development and infectivity of new cells

One of the aspects of the developmental cycle of *A. marginale* that is not fully understood is the mechanism by which the organism infects new cells. This has become particularly important in regard to attempts to stimulate increased growth and development of the organism *in vitro*. It has been accepted that infection of erythrocytes is initiated by a single initial body. It has been demonstrated with immunofluorescence that infection may occur between erythrocytes *in vitro* (Kessler and Ristic, 1979) but morphological evidence of transfer has not been shown. During log phase increases in parasitaemia at levels as high as 70% we have not been able to identify the exit of parasites from infected erythrocytes in samples collected directly from reacting animals or in short-term cultures. In these samples it is not uncommon to find single initial bodies which have just entered a cell and very rarely an initial body outside of a cell. The actual infection of organisms may not occur in general circulation but in the close confines of the microcirculation. It has been a consistent observation that organisms free from host cells appear to lose morphological integrity rapidly. Closer contact between cells would be enhanced in areas such as capillary beds and transfer might be facilitated. Another observation in short-term cultures initiated with cells in log phase parasitaemic growth was an apparent disassociation of intracellular inclusion bodies. Inclusions in various stages of breaking apart from the inclusion body membrane have been consistently observed in samples cultured for 24–48 hours and from samples collected directly from reacting animals. Exit of individual initial bodies, free of the inclusion membrane, might be accomplished with less damage to the erythrocyte membrane than if the entire inclusion body functioned in the exit process. This mechanism is still being investigated.

Manipulation of intercellular movement

The observation that infection of uninfected erythrocytes ceases after 24–48 hours in the various culture systems investigated suggests that one or more factors present in the vertebrate host are not present *in vitro* or are used up rapidly. Factors which might enhance the entry or exit of *A. marginale* are being investigated. Calcium has been shown to play an important role in the long-term maintenance of other intraerythrocytic parasites *in vitro,* particularly during invasion and early growth (Tanabe *et al.,* 1989; Krishna and Squire-Pollard, 1990; Ray *et al.,* 1989). The adhesion of chlamydial organisms to host cell membranes and enhanced infection rates of these cells have been associated with a flux of calcium across the cell membrane (Murray and Ward, 1984).

In our studies a calcium influx into cultured erythrocytes infected with *A. marginale* was induced using the ionophore A23187. Cultured erythrocytes from two animals with rising parasitaemias were treated with 10 uM of A23187 in the initial trial. After 24 hours, there was a decrease in the percentage of infected erythrocytes (PEI) in both the treated cultures (from 19 to 12% and 13 to 7.7%). The untreated control cultures increased slightly over 48 hours. In the second trial, infected erythrocyte (19%) cultures were treated with ionophore at 1 uM and 5 uM on three consecutive days. Two controls were used—untreated infected erythrocytes and infected erythrocytes treated with the same volume of dimethylsulphoxide (diluent for the ionophore). Infection levels were monitored and haemoglobin samples were taken 24 hours after each treatment. Intracellular calcium levels were found to increase following addition of ionophore but there was no significant difference between the 1 uM and 5 uM samples. There was a slight increase in PEI from 19 to 22.6% over 48 hours in untreated cultures, a slight drop from 19 to 18% after 24 hours in cultures treated with 1 uM and a significant decrease from 19 to 13.8% after 24 hours in cultures treated with 5 uM of A23187. Cultures treated with the diluent (DMSO) only increased slightly from 19 to 20.75% after 24 hours. Haemoglobin levels were considered low in all samples but slightly higher levels were found in treated culture supernates. Levels were not sufficient, however, to incriminate lysis of the more fragile infected cells as the cause of the significant drop in PEI in cultures treated with 5 uM of ionophore.

Electron micrographs of culture samples revealed what appeared to be the expulsion of entire inclusion bodies as well as individual initial bodies from the periphery of erythrocytes during the first 24 hours in ionophore-treated cultures and may have been responsible for the decrease in PEI. In most of these observations erythrocyte membranes appeared to remain intact following the release of organisms. While infection of erythrocytes did not appear to be enhanced, release of organisms was facilitated. Further studies will utilize intracellular calcium chelators to determine if release was due to action of the ionophore or a calcium-dependent mechanism.

Culture systems using tick-derived stages of *Anaplasma marginale*

The developmental cycle of *A. marginale* in ixodid ticks has proven to be extremely complex. Research using male *D. andersoni* transferred from infected to susceptible cattle has demonstrated a sequence of development in which the organism first undergoes growth in midgut epithelial cells (Kocan *et al.,* 1980, 1988, 1990a, 1991). This is followed by an intermediate site of development in gut muscle cells (Kocan *et al.,* 1990a) with the final stage of development in salivary gland cells (Kocan *et al.,* 1988, 1991; Stiller *et al.,* 1989). It was also shown that different morphological forms occur in the tick tissues and that not all of these forms are infective for cattle (Kocan, 1986). Long-term studies on the co-ordinated development of this organism in *D. andersoni* ticks (Kocan, 1986; Kocan *et al.,* 1990b) have resulted in feeding regimes in which consistently high levels of organisms can be obtained from each tick tissue. This ability forms the basis for present efforts at initiating continuous culture systems.

Initiation of erythrocyte cultures with tick stages of *Anaplasma marginale*

A hypothetical life cycle for *A. marginale* involves a tick-derived stage entering erythrocytes of the vertebrate host by endocytosis and subsequently undergoing binary fission (Ristic and Watrach, 1963). The infection of bovine erythrocytes by a tick-derived stage of the organism has not been demonstrated previously. In an attempt to initiate an *in vitro* infection with a tick stage and show direct infection of bovine erythrocytes, cultured erythrocytes were inoculated with the salivary gland stage of *A. marginale*. Uninfected male *D. andersoni* were fed on a clean calf to serve as controls. After holding ticks for five days, each set was fed on separate susceptible animals for 14 days. Ticks were dissected, salivary glands were removed and homogenized. Inoculum prepared from 10 ticks was added to each 35×10 mm culture dish containing erythrocytes in medium supplemented with 20% foetal calf serum. Samples were collected after 30, 60 and 120 minutes for electron microscopy (EM). After 24 hours, samples were collected daily for EM, Giemsa and FITC staining. Salivary glands of ticks fed on the infected animal were found to contain *A. marginale* colonies and the animal subsequently developed anaplasmosis. Colonies and individual rickettsiae were found in EM samples of the inocula from these ticks. No rickettsiae were found in control ticks and the calf on which they were fed did not become infected with *Anaplasma*. In EM samples from the first day, rickettsiae were observed entering erythrocytes by an endocytosis-like process. Individual organisms were observed intercellularly throughout the observation period but no replication was observed. No rickettsiae were observed in control samples. Due to the size of individual rickettsial bodies identification with light microscopic staining is difficult to interpret. Although replication did not appear to occur, infection of cells can be achieved. Present efforts are concentrating on increasing infection levels in the system and if sufficient levels can be achieved it may have application in *in vitro* neutralization studies.

Infection of nucleated cells with tick stages of *Anaplasma marginale*

Hidalgo *et al.* (1989a) first used tick gut stages of *A. marginale* to infect an embryonic tick cell line and indicated that replication may have occurred over an 11-month culture period. It has been suggested that this organism may require actively growing cells and that initial development is associated with the host cell nucleus (Hidalgo *et al.,* 1989c). Investigations are presently under way to infect different nucleated cell lines of arthropod and vertebrate origin with the different tick-derived stages of *A. marginale*. Initial attempts to infect bovine endothelial cell lines with gut stages have been inconclusive. Similar cell lines inoculated with salivary gland-derived stages have been found to be positive using the polymerase chain reaction after six weeks post inoculation after several passages. *Anaplasma*-like inclusions similar to those reported by Hidalgo (1989c) were also observed with light and electron microscopy. Infectivity of cultured material is being investigated. In preparing viable inocula from tick tissues two consistent observations have been made. It is difficult to separate individual organisms from colony membranes and associated tick cell material and, once free of inclusion membranes, organisms appear to lose viability quickly. Techniques to prepare cleaner inoculum and facilitate contact between host

cells are being evaluated. Of particular interest are events which occur just after inoculation. If survival of organisms occurs, the intracellular events need to be determined and these are under investigation.

CONCLUSIONS

The precise requirements for growth of *A. marginale* have not been fully defined. The parasite appears to survive in several *in vitro* situations but replication has not been confirmed. Use of short-term erythrocyte cultures has proven useful in studying certain aspects of the behaviour of *A. marginale*. If the infection of erythrocytes with salivary gland stages can be optimized, this system would be beneficial in studying neutralization of infection. Emphasis is presently being given to the use of tick-derived stages of *A. marginale* in various nucleated cell lines. Based on results to date this appears to be the most promising area to pursue.

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SUMMARY OF DISCUSSIONS

Summary of discussions

Disease distribution, economic importance and control

Chairman: Dr. R. Dalgliesh
Rapporteurs: Dr. B.D. Perry, Dr. A.W. Mukhebi

BABESIOSIS AND ANAPLASMOSIS

The discussion centred on three areas: the effects of endemically stable states on livestock production; the effects of different strains and intensity of *Anaplasma marginale* challenge on the efficacy of *A. centrale* immunization; and the changing incidence of vaccinal failures in the face of live *Babesia bovis* vaccine use in Australia.

Unpublished studies from Uruguay suggest considerable losses in live-weight gain in apparently healthy but carrier animals, but this work has not been substantiated elsewhere. Evidence of differences in weight gain between vaccinated and control cattle in endemically stable areas was also reported. However, animals immunized with *A. centrale* in Australia experienced some decrease in live-weight gain, but this was followed by compensatory growth over the subsequent two to three months. In immunization trials in Malawi, there were no differences in live-weight gain, body score and packed cell volume between vaccinated and control animals six months to three years old two months after trivalent *(A. centrale, B. bigemina* and *B. bovis)* immunization.

The variation in clinical response to *A. centrale* immunization was emphasized. *Anaplasma centrale* has a behavioural difference from *A. marginale,* and a transmission difference, being transmitted by *Rhipicephalus appendiculatus simus* and not by *Boophilus* spp. It was noted that in some Asian countries *Anaplasma* had a more central position within the erythrocyte, although they were called *A. marginale.* They were reported from areas in which *A. centrale* vaccination may have been used. Some regional differences to immunization with *A. centrale* were noted. Some alarming reactions were reported from Australia. The role of differing *A. marginale* challenge was discussed. DNA probes had shown a homogeneity in Australian *A. marginale* isolates, whereas a heterogeneity in North American isolates was reported. It was suggested that with only one recorded introduction of *A. centrale* to Australia, there was no heterogeneity in isolates in that country, and the clinical disease also showed marked homogeneity.

Vaccinal failures with *B. bovis* vaccine in Australia were originally blamed on the strain used. Although after 1978, a frozen stabilate from an early passage was used, failures still persisted. Failures seemed to spread from one property to another and tended to be associated with frequent vaccine use. However, when breakthrough strains were isolated in the laboratory and tested against the

vaccine strain, it was shown that the protective effect had not changed. This indicates changes in the field populations of *B. bovis* rather than the vaccine strain.

It was evident from the discussions that there are inadequate data to make comprehensive and practical estimates of the economic losses attributable to these diseases and the benefits from their improved control.

COWDRIOSIS

The economic importance of heartwater in comparison with other tick-borne diseases and the difficulty associated with quantifying this was discussed at length. It was stressed that there are considerable differences throughout Africa in disease occurrence depending on cattle types and acaricide use. These differences influence the prevalence of endemic instability. It was suggested that in South Africa, Zimbabwe and West Africa, the disease was considerably more important than other tick-borne diseases. It was also identified as a major problem in Kenya where other tick-borne diseases were controlled by immunization under field-trial conditions. It was emphasized that heartwater has been considerably under-diagnosed due to the lack of a diagnostic test for use in the live animal.

The outstanding questions arising from the discussions on anaplasmosis, babesiosis and heartwater were:

In general
- Are there better ways to define the effects of babesiosis and anaplasmosis on livestock production? Are control measures justified? If so, what control measures are economically feasible?
- How variable is *A. marginale*? What are the reasons for difference in virulence among countries and where can *A. centrale* be useful, and where not?
- Does repetitive vaccination in a particular situation lead to selection for an emergence of antigenetically different, virulent parasite strains? If so, what are the mechanisms and what are the solutions?

In South America
- Why is the American *A. marginale* apparently so much more virulent than the same parasite in other countries? Why does *A. centrale* not protect?
- What can be done to stop the spread of *A. marginale* by mechanical transmission in vaccination programs for other diseases such as foot-and-mouth disease?
- Is the inactivated culture vaccine sufficient for control of babesiosis in Venezuela and other South American countries? Should live vaccines be prevented?
- Why is *B. bigemina* a particular problem in Brazil?

In West Africa
- Is the introduction and use of *Anaplasma* and *Babesia* vaccination justified in Nigeria and other countries in the West African region?
- Is it possible that *Boophilus decoloratus* is the vector of *B. bovis* in some parts of Nigeria? If not, what is the reason for the epidemiological evidence that suggests that this is so?

In Southeast Asia
- Can we develop an internationally coordinated approach to ensure all susceptible cattle imported into tropical/subtropical areas are adequately protected against babesiosis and anaplasmosis?
- Why was the Sri Lankan strain of *B. bovis* not amenable to a reduction in virulence by passage whereas it has been successful on at least 10 strains in Australia, South Africa and South America? What are the implications for other countries?

On cowdriosis
- Are there better ways to define the effects of heartwater on livestock production? Are control measures justified? If so, what approach is economically feasible? The importance of defining the effects of heartwater on production cannot be over-emphasized. Ranking of the economic importance of the disease must be an integral part of research programs. Heartwater is apparently top priority in many countries and it is, therefore, wrong to push programs on other diseases ahead of heartwater.
- How great is the threat of spread of heartwater in other parts of the world where it is a relatively new disease, and how best can this threat be met?
- What is the potential of the tick-decoy approach in the control of heartwater? If it is extremely effective for vector control, it may have commendable economic advantages over other control methods.

Diagnosis

Chairman: Prof. G. Uilenberg
Rapporteurs: Dr. P.A.O. Majiwa, Dr. O.K. ole-MoiYoi

The discussants agreed that infectious organisms needed to be accurately identified. This called for the use of reagents which achieve the objective precisely and quickly. It is extremely important to convince farmers of the simplicity and reliability of a particular test because they are the ones who must pay for the services of a veterinarian to treat their animals. They therefore need to be convinced that the test really works. The farmer and the veterinary clinician need tests that are quick and reliable and which will thus identify infected animals that need treatment before their condition worsens. A point that was emphasized is that it would be desirable to have a diagnostic test to detect a parasite before the onset of disease, before it is too late

to successfully treat the animal so that it regains full productivity. The reagents used should help identify infected animals, the strain or substrain (species or subspecies) of the infectious organism, and should also identify those organisms which do not respond to treatment, the so-called `breakthrough' strains.

It was clear that microscopic examination of blood smears might be insufficient in many situations. Although a microscopic examination can be done routinely and quickly, it can also be subjective. This emphasized the need for reagents upon which standard and objective tests can be based. Recombinant antigens which can be standardized for use in ELISA, other antigen capture assays and DNA hybridization probes could meet these requirements. Indeed, for some of the organisms, such as *Anaplasma,* both reagents are available and what needs to be done is to use the reagents to answer specific epidemiological questions.

It was pointed out that *Anaplasma* infections can be sterilized; however, animals usually remain carriers after treatment. It is therefore important to have assay systems which will discriminate between acutely infected, subacutely infected and carrier animals. It is apparent from the above that a combination of diagnostic tests will have to be employed in many situations, particularly since in these infections, antibody titres may bear little, if any, relationship to the degree of immune protection of the animals. None of the reagents available, recombinant DNA probes or serological reagents, has been critically evaluated as to specificity. To do this, one would need to perform expensive experiments that involve the use of many animals and/or appropriate vectors of the infectious organism. Questions also arose as to the necessity of identifying infectious organisms present either in the vectors or in the hosts at such low levels that they would not cause disease or be transmitted. This important issue was not resolved in the discussion.

New vaccines

Chairman: Dr. A.J. Musoke
Rapporteurs: Dr. L.L. Logan-Henfrey, Dr. D.J.L. Williams

The discussion focussed on three topics—the nature of the immune response, the role of the carrier state and the importance of innate resistance in local breeds.

ANAPLASMA MARGINALE

The relevance of the multiple copies of the 29 amino acid tandem repeat was questioned. So far all isolates have shown multiple copies of this sequence repeat although the number of repeats varied between isolates. All isolates tested reacted with a MAb raised against this amino acid sequence, suggesting that the motif is conserved. The major surface proteins (MSPs) were found to be protective in preliminary trials in Zimbabwe. A point was made that challenges should be made with sporozoites to evaluate the level of protection against tick challenge in the field.

A general question about vaccine delivery systems was raised. Vaccinia virus is currently being evaluated as a vector in the US. This allows for 2 conformational changes in expressed proteins but stimulates a cell-mediated immunity rather than an antibody response. Other adjuvants such as biodegradable plastics are being developed.

COWDRIA RUMINANTIUM

A question was raised concerning the CR32-kDa antigen's ability to elicit a protective immune response. *In vitro* neutralization assays are being carried out and preliminary experiments showed that mice immunized with affinity-purified CR32 in Freud's complete adjuvant were not protected. It was postulated that if this antigen were protective, selective pressure would have resulted in polymorphisms within the protein. This does not appear to be the case. The group working on this protein agreed that its protective capacity was in doubt but suggested that it could be a very useful diagnostic tool.

One discussant thought that the nature of resistance in local livestock populations to a variety of diseases should be studied in depth. Questions were raised regarding the level and seasonality of tick challenge in endemic areas and its role in maintaining resistance. Similarly the question of whether resistance was related to an animal's carrier status was discussed. No data are available to answer these questions.

Finally the discussion returned to the type of immune responses which were generated by infection or vaccination. It was agreed that the type of protective immune response generated by infection should be characterized since the epitopes involved in cytotoxic lymphocyte responses would be very different to those which elicit antibody responses. In addition the antibody response generated by single-shot vaccines should be characterized to determine not just the titre but the avidity and the rapidity of the response. In other words, can it outstrip parasite multiplication?

Natural immunity may be different from that which can be induced artificially (for example, in malaria, sporozoite antigens contain no T-cell epitopes). By adding T-cell epitopes, effective antibody responses can be generated. With the isolation of genes for the protective antigens of *Babesia, Anaplasma* and *Cowdria,* elegant manipulations are being carried out, allowing the design of effective subunit vaccines.

In vitro cultivation

Chairman: Dr. H. Hirumi
Rapporteurs: Dr. R. Kaminsky, Dr. P.R. Spooner

It was emphasized that the *Babesia* culture system is well established and suitable for the *in vitro* isolation of field strains. Areas for further improvement were considered to be the development of (1) an automated system to reduce laborious medium changes, (2) large-scale culture systems and (3) methods to cultivate the parasite stages in the tick. Due to lack of knowledge of the development of *Babesia* in ticks, only limited emphasis has been placed on the culture of the arthropod life cycle stages.

COWDRIA RUMINANTIUM

Improvement of the culture system for *Cowdria* is still desirable, in particular with respect to the growth rate of endothelial cells. The source (tissue origin) of endothelial cells may have an influence on the growth of the organisms. Problems in the cryopreservation of *Cowdria* may be overcome by the use of sucrose-gluta-mate-phosphate buffer and by using culture material two days after infection. It was suggested that inconsistent DNA recovery was due to the difficulty in disrupting elementary bodies which may be improved by using reducing agents such as 2-mercaptoethanol or dithiothreitol. The success rate for initiation of cultures using plasma was considered to depend on the state of infection in the animal. Problems in the propagation of the arthropod life stages were not discussed.

ANAPLASMA MARGINALE

The precise requirements for growth of *A. marginale* have not been fully defined for either bovine or tick stages. The parasites can be maintained in culture but the present systems have not been shown to maintain infectivity or to support replication. Short-term cultures can be used for studies only on certain aspects of the biology of *Anaplasma.* Long-term cultures are obviously needed for chemotherapeutic and immunological studies.

Commercialization

Chairman: Dr. T.T. Dolan
Rapporteurs: Dr. J.A. Lawrence, Dr. N. McHardy, Dr. G. Bissuel

A separate discussion was held on the likely contribution of the pharmaceutical industry to the control of tick-borne diseases.

THERAPEUTICS

Drugs exist for control by therapy and prophylaxis of babesiosis, anaplasmosis and cowdriosis, but they are few and some previously useful products are no longer available. The drugs available for the treatment of babesiosis are diminazene acetu-rate (Berenil, Ganaseg) and imidocarb dipropionate (Imizol). In addition, tetracycli-nes are increasingly used for specific roles such as the control of vaccine reactions. The drugs that have been withdrawn are amicarbalide, phenamidine and pentamidine. Quinuronium sulphate (Acaprin) is not available in most markets but is a mainstay, particularly for ovine babesiosis, in the Middle East. Two drugs are available for anaplasmosis—tetracyclines and imidocarb. Users appear reasonably satisfied with their efficiency. The only available drugs for cowdriosis are oxytetracycline and its near relative doxycycline, which shows promise as a sustained-action product.

Two active leads, rifampins and thiosemicarbazones are not expected to become available for regulatory/safety reasons.

No new therapeutics for these three diseases are believed to be in development. This is a cause for concern since, although drug resistance among these diseases is not a significant problem, in the long term it is inevitable and new products will be required. The withdrawal of drugs has been due to declining markets, presumably because currently available products are superior, and not because of the development of resistance. Nonetheless, some would still find niche uses if they were available.

If new therapeutics are to be developed, this can only be done by industry. The cost of developing new therapeutics solely for tick-borne diseases is regarded by industry as prohibitive. Apart from the difficulty of identifying new active compounds, the need to establish human safety in the face of increasingly stringent regulations is the principal cost constraint.

While no specific need for new therapeutics was identified, the possibility of developing sustained action products such as doxycycline for control of cowdriosis would be attractive. The problem of human safety from persistent residues could, however, pose insuperable regulatory problems.

Any new product would inevitably be relatively expensive because of the need to recoup the cost of its development (perhaps £50 million over 10 years) and to give a return on investment.

DIAGNOSTICS

A number of improved diagnostic tests have been developed by research organizations to a level at which they could be of value for epidemiological surveys or clinical diagnosis. There is little, if any, interest from commerce in further development and production of these tests for field use because of the limited perceived market. Diagnostics will almost certainly require public funding for development. FAO/IAEA and OIE have already initiated development and production of a number of tests. It was the general opinion that these organizations were the most appropriate for this and for monitoring quality, and were in the best position to seek funding from donors and commerce to support the work. Commerce should be encouraged to sponsor development of diagnostics to improve the utilization of vaccines and chemotherapeutics in the field.

VACCINES

There is an urgent need to increase the efficiency of cattle production in Africa. The population is increasing at a rate of three percent, and it is predicted that by the year 2025 the requirement for red meat will increase three to four times. One of the major constraints to improved production is tick-borne diseases, and vaccines are the most desirable way of controlling them. Great progress is being made in the development of subunit vaccines but it is unlikely that any will be on the market within the next five to seven years. In the meantime, every effort should be made to promote the use

of conventional vaccines, despite their limitations. This will require some input from public funds and the involvement of commerce.

Cattle owners in Africa are generally accustomed to paying for acaricides and chemotherapeutics, but in the past vaccines have often been provided free. There is therefore a reluctance to pay for vaccines for tick-borne diseases, which must be overcome. Widespread use of vaccines will require a concerted extension approach and good marketing organization. Commerce should be encouraged to develop packages incorporating vaccines, acaricides and chemotherapeutics, which can be marketed effectively to improve productivity. Farmers should be given better access to credit to encourage them to purchase such packages to initiate an economic approach to production which is essential for livestock improvement.

If significant improvement in livestock production is to be achieved, it is essential to develop strategies for integrated tick and tick-borne disease control and to promote them by intensive extension programs. If successful, this will have the effect of improving the quality and value of cattle in Africa, which in turn will increase the market for vaccines and other products and therefore increase the motivation for commerce.

Concluding discussion

Chairman: J.J. Doyle
Rapporteur: T.T. Dolan

The discussion on babesiosis returned to the development of resistance to the *B. bovis* blood vaccine in Australia and the selection for parasite populations in the field which differed from the vaccine strain. There was no evidence (yet) of problems in other countries where the Australian vaccine had been used, but this may simply be because repeated vaccination has not been applied. Should resistance develop, it will be necessary to change the vaccine strain as was done in Australia. In the case of vaccination against *A. marginale,* it was apparent that *A. centrale* worked inconsistently and the use of *A. marginale* needed to be explored more widely to provide comparative data and perhaps a better vaccine alternative in problem areas.

In South America there is a large market for the current anaplasmosis and babesiosis vaccines but their availability is a problem. A culture supernatant vaccine for babesiosis is being tested in a large number of cattle and while the results are generally positive, there are difficulties in interpreting them. *Babesia bigemina* is most important in Brazil while *B. bovis* is the more important pathogen in most other countries. It is important to be able to determine the relative importance of the two species and to study the factors influencing virulence.

The vast majority (90%) of cattle in West Africa are owned by pastoralists and hand de-ticking and acaricides are the most widely used control measures. Studies on the vectors for *B. bovis* were required and integrated control measures, which might include the cautious introduction of vaccination, should be considered.

In regard to Southeast Asia, the point made on an internationally coordinated effort to ensure that susceptible high-grade cattle being introduced into tropical and subtropical countries were protected against anaplasmosis and babesiosis was repeated. Organizations such as OIE could be responsible for ensuring that this is done as the high losses encountered in these introduced cattle totally undermined this method of livestock improvement. The failure to attenuate Sri Lankan *B. bovis* strains could indicate that similar problems will be encountered elsewhere. This strengthens the case for importing well-characterized, safety-tested vaccines rather than attempting to develop vaccine strains in a multitude of countries.

There is good evidence for the spread of *Amblyomma* species in a number of countries and, depending on the region of Africa, this has important implications for cowdriosis and/or dermatophilosis. The potential of the tick decoy method of control of this species based upon a pheromone elaborated by the attached male tick and insecticide was considerable. The possibility of using this ingenious approach in a plastic tag slow release (pheromone and insecticide) form could be an important practical control measure.

Much greater emphasis is needed to determine the economic importance of tick-borne diseases. The costs of acaricides, dip construction, maintenance and use have been estimated for many countries but particular attention must be directed towards an accurate assessment of mortality and of the production losses to subclinical disease. Having accurate estimates of these losses could induce pharmaceutical companies to re-appraise the potential for investment in tick-borne disease chemotherapy, diagnostics or vaccines. A start should be made and methodologies developed which would be applicable in many situations. The use of existing models or the development of new models for studies of disease, economics or control would contribute to faster and more accurate assembly of these data. The availability of hard data on the economic impact of these diseases would also strengthen the case for national and donor investment in their control.

The development of high-quality standardized diagnostic tests is critically needed for epidemiological studies. The development of tests for immunity, for presence of infection (carrier state), for discrimination between field and vaccine exposure, for identification of strains and for detection and quantification of infection in vectors is desirable. Such tests would greatly improve epidemiological studies and contribute to the assessment of economic impact, particularly in assessing the effects of subclinical infections. It was agreed that a group representing the institutes attending the workshop would meet to establish collaboration for the development of improved diagnostics, particularly antibody- and antigen-detection ELISAs for anaplasmosis and babesiosis.

The potential for subunit vaccines for the tick-borne diseases was very promising. In the case of *Anaplasma,* partial protection was provided against parasitaemia, but not anaemia, using recombinant antigens of MSPs. For *B. bigemina,* polypeptide complexes from the surface of merozoites isolated by affinity chromatography have been shown to provide up to 70% protection. Recombinant antigens of *B. bovis* based upon fractionated lysates also provided protection, which was improved when they were combined. Culture supernatant vaccines being explored for a number of *Babesia* species also promise well. However, these novel *Babesia* vaccines all perform less efficiently than the live vaccines available and therefore

will require considerable improvement before they can replace them. In the case of *Cowdria,* it was generally believed that the important immune responses were cell-mediated. The identification and isolation of the expressed peptides may be difficult and ILRAD's immunological expertise could contribute to the search for protective antigens. Although vaccines based upon infection and treatment or tissue culture are effective, they are compromised. The development of subunit vaccines is a high priority research need so that live vaccines with the attendant risks of transmission of other pathogens, the introduction of vaccine parasite strains, the possibility of reversion to virulence, and the complications of delivery in many tropical countries may be replaced.

The questions of challenge dose and the nature of challenge were also raised in the testing of protection provided by novel vaccines; what parasites (homologous or heterologous) should be used and should blood, tissue culture, stabilates or ticks be used and what dose? Finally, when subunit vaccines are produced, what international approval/ regulations will be required prior to their release, especially if live delivery systems are used.

The major points regarding *in vitro* culture and commercialization had been discussed thoroughly in the sessions immediately prior to this concluding discussion and there was no further discussion.

Concluding remarks

Dr. Gray thanked the participants for the quality of their papers and for the openness of the discussion. They had provided ILRAD with a comprehensive update on the research and control of the three diseases. This information will greatly assist ILRAD in developing research strategies for the next ten years. Dr. Gray strongly supported the setting up of collaboration for development of standardized diagnostic assays. He thought that ILRAD's future involvement in other tick-borne or tick-associated diseases, if approved by the Technical Advisory Committee of the CGIAR, would be in the form of collaboration, exploiting ILRAD's advantages in bovine immunology, vaccine development, location in Africa and its excellent laboratory and cattle production facilities. He thanked the organizers, chairmen and rapporteurs and hoped that the publication of the proceedings would be timely and useful.

CONCLUSIONS
AND RECOMMENDATIONS

Conclusions and recommendations

- The epidemiology of tick-borne diseases is poorly understood in most countries of the world. It is recommended that detailed structured studies be conducted within national and regional programs that would monitor vector distribution and dynamics, the interplay of the different parasites and their virulence, and carrier states so that the epidemiology is understood and appropriate control measures can be applied.

- The variation in quality and frequent unavailability of diagnostic tests for tick-borne diseases seriously compromise epidemiological studies and disease surveillance. It is recommended that reproducible and simple-to-use tests should be developed and that the groups attending this Workshop should meet to establish a collaboration to address the development, production and distribution of these tests.

- The effects of tick-borne diseases on the productivity of cattle are widely recognized but poorly quantified. It is recommended that studies on the productivity of cattle should be incorporated from the planning stages of future epidemiological and control programs for tick-borne diseases at both national and donor level.

- Short-interval acaricide application as a control measure for ticks and tick-borne diseases is not being employed effectively in many countries and alternative control measures are required. It is recommended that integrated control, which might include strategic acaricide use, exposure of young cattle to natural challenge, immunization with available vaccines and chemotherapy, be adopted. The use of the tick hormone/insecticide decoy method for *Amblyomma* control should be tested more exhaustively as a potential component within these integrated measures.

- Live vaccines for tick-borne diseases have well-known limitations but for many countries they represent the only available means of disease control. It is recommended that where vaccines are used, strict attention be paid to quality control and safety testing and if this cannot be guaranteed, adequately tested vaccines should be obtained from established laboratories.

- The distribution of *Amblyomma* ticks, vectors of *Cowdria ruminantium,* is expanding in a number of countries in Africa and in the Caribbean. It is recommended that strict monitoring of the changing distribution of these ticks be undertaken and the possible spread of heartwater into susceptible populations of livestock be assessed.

- The available chemotherapeutics for tick-borne diseases are effective and resistance has not been recorded. However, important effective drugs have been withdrawn from the market and the high cost of developing new veterinary drugs inhibits further investment by industry. It is recommended that drugs be administered at the correct dose rates and that resistance be regularly monitored.

- The benefits of the introduction of high-producing cattle into many tropical countries is seriously undermined by mortality and morbidity due to tick-borne

diseases. It is recommended that where possible such cattle be immunized against *Anaplasma* and *Babesia* before introduction. Organizations such as OIE should inform recipient countries of the high susceptibility of these cattle to tick-borne diseases and of the vaccination options available.

- The current research on subunit vaccines for *Anaplasma* and *Babesia* shows considerable promise of success. It is recommended that further investment be made in attempting to identify and characterize potentially protective antigens for future vaccines and that ILRAD's experience in cell-mediated immunity be drawn upon in the search for antigens of *Cowdria.*

- *In vitro* cultivation of tick-borne pathogens is an important technique that has contributed to identification of anti-parasitic drugs, understanding of the basic biology of parasites, provision of DNA for molecular studies and antigens for diagnosis or immunization. It is recommended that studies be continued towards the production of automated, bulk culture systems for *Babesia,* the identification of more rapidly growing endothelial host cells for *Cowdria,* the development of long-term culture systems for *Anaplasma* and the development of arthropod cell culture systems for the production of livestock-infective stages of all three parasites.

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