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R E P O R T

INTRACELLULAR BLOOD PROTISTA  
WITH REFERENCE TO  
IMMUNOPROPHYLAXIS AND CONTROL

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Consulting Report

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## I. OBJECTIVES OF THE SUB-PROJECT

This subproject focuses on the development and implementation under laboratory and field conditions of immunoprophylactic and diagnostic methods needed for prevention and control of three major tick transmitted protistan blood diseases of food producing animals e.g. cattle, sheep and goats and working animals such as horses. Protistas are defined as intracellular obligate parasites that are capable of parasitizing various blood cells, in the case of this subproject, erythrocytes of host animals. The three diseases and their causative agents of relevance to this subproject are: Theileria annulata, causative agent of theileriosis; Babesia bovis and B. bigemina, causative agents of babesiosis; and Anaplasma marginale causative agent of anaplasmosis. In addition to food producing animals, draft equine species, (e.g. horses, mules and donkeys) are also highly susceptible to infections with Babesia parasites, such as Babesia equi and B. caballi, and suffer severe losses when infected by these agents. Similarly, dogs which are important within the police and military forces are known to experience severe infections and mortalities due to babesia agents.

Appropriate research, including recent advances in bio-technology and cellular engineering involving antigen/antibody systems, is needed to develop method(s) for the control of theileriosis. With reference to babesiosis and anaplasmosis, scientists at the University of Illinois have developed by use of modern cellular engineering techniques, serodiagnostic procedures and vaccines for prevention and control of bovine and canine babesiosis and bovine anaplasmosis. Hence, transfer of diagnostic and vaccine technologies for babesiosis and anaplasmosis from the University of Illinois to India for testing under laboratory and field condition is needed. Such a transfer should be accomplished by contractual arrangement between the University of Illinois and ICAR-AID and by a training program as proposed under this subproject.

## II. PARTICIPATING UNITS AND ADMINISTRATION OF THE SUBPROJECT

The study under this subproject is being conducted at five Indian institutions. These are: Department of Medicine, Haryana Agricultural University, Hissar; The Division of Parasitology, Indian Veterinary Research Institute, Izatnagar, U.P.; The Animal Disease Diagnostic Laboratory, National Dairy Development Board, Anand, Gujarat; The Department of Veterinary and Animal Sciences, Punjab Agricultural

University, Ludhiana, Punjab; and the Department of Parasitology, Madras Veterinary College, Tamil Nadu Agricultural University, Madras. Very recently, Patna College of Veterinary Medicine at Bihar joined the program of this subproject.

The Coordinating Unit and Central Laboratory for the subproject are located in Hissar under the direction of Dr. M.N. Malhotra of ICAR.

### III. REVIEW OF RESEARCH ACTIVITIES AT PARTICIPATING STATIONS

The consultants were briefed on the research progress made at each station at a two-day workshop held at Hissar, by consultation with various research groups at Delhi and by visits to Anand, Madras and Ludhiana. Some important research developments that took place during the past year at Punjab Agricultural University will be described in the next section (IV) while accomplishments of other stations are summarized here.

With very minor exceptions, the research at the participating stations is basically limited to the development of preventive measures against bovine theileriosis. In this effort two principal research

approaches are being used: (1) High passage lymphocyte cultures of T. annulata and (2) Use of tick-derived sporozoites in combination with chemotherapy.

In the first approach using high passage infected lymphocyte cultures each of the three stations (IVRI, HAU and NDDB) claim obtaining promising results. Based on the number of cattle used, including some newly imported highly susceptible cattle, NDDB seems to have made more advanced progress. These findings can be greatly strengthened by the availability of clear evidence that the vaccine recipient animals were fully susceptible to infection with T. annulata. For this purpose, there is a need for the development of an accurate and sensitive diagnostic test. Such a test has now been developed (see section IV below). This test can now be used to monitor immune responses following vaccination and challenge of experimental animals. The pattern of immune response obtained by experimental challenge should then be related to data generated under natural challenge exposure.

In the second approach for prevention of bovine theileriosis, susceptible young animals 1 to 2 weeks of age are being inoculated with tick-derived sporozoites. This procedure is followed by application of

chemotherapeutic treatment. While this infection and treatment method (premunization) may not be a useful procedure for mass field use, it may be useful to compare protective effects induced by it with those using cell lymphocyte culture derived schizonts.

#### IV. MAJOR RESEARCH DEVELOPMENT AT PAU

Very recently (last 10 months), thanks to the collaboration between the consultant and the research team of Punjab Agricultural University led by Dr. A.S. Grewal, a major break through has been achieved in the development of serodiagnosis and immunoprophylaxis against bovine theileriosis. Scientists working on this disease in various regions of the world, including India, have traditionally utilized, with various inconsistent results, in vitro produced lymphocyte phase cultures of the organism as a source of a potential vaccine. By following this consultant's work with Balesia, Grewal and his team developed a new in vitro system in which the blood phase of theileriosis (piroplasms) were utilized as a source of diagnostic and vaccinal material.

Using blood phase piroplasm as antigen, PAU developed a rapid and highly sensitive DCT-ELISA test for detection of anti T. annulata

antibodies and consequently diagnosis of theileriosis. This test is currently being used in the field seroepidemiologic studies. Some 134 of the private dairy farms registered with the Punjab Dairy Farms Association plus 8 semi-Government Cross-bred Dairy Farms are being covered in this study. The selected Dairy Farms represent widely different regions of the Punjab state covering both cross-bred cattle and buffaloes in these organized herds. The industrial significance of data to be generated in this new epidemiologic study will be to provide information on prevalence rates of new infections in reference to the season, correlation of infectivity with production performance of cattle, role of buffaloes as reservoirs of infection and the assessment of the efficiency of the vaccine.

For the development of vaccinal material for prevention of theileriosis, Grewal and his team utilized primary cell cultures initiated from the blood of infected cattle. In this sequence they collected whole blood from the jugular vein of infected cattle. Using the buffy coat of this blood, they established in vitro macrophage - lymphocyte co-cultures. From these cultures, similar to this consultant's work on Babesia, they harvested cell free material present in the supernatant of the culture medium. Preliminary studies showed that the supernatant medium contained cell-free Theileria piroplasms.

This piroplasm material was injected into a susceptible animal. The animal developed a serologic response that was monitored by the DOT-ELISA. The animal showed no clinical signs of the disease and upon challenge of this animal with tick-derived sporozoites it was found to be fully protected.

This unique new system of generating vaccinal and diagnostic antigens of *T. annulata* has many significant potentials. Aside from its diagnostic and vaccinal efficacy, the advantage of this new method is its simplicity and cost effectiveness. In addition, the method of antigen production can be scaled-up to accommodate industrial levels for all India vaccine production. Finally, this novel vaccination method has a very important strictly biological significance. The blood phase of theileriosis represents the final growth stage of *T. annulata*. Blood piroplasms which developed at this stage are being utilized by ticks as a source of infection and consequently further perpetuation of the agent and spread of the disease. By use of immunization against blood phase piroplasms the cycle of further spread of the disease by ticks can be blocked. This in turn should result in gradual reduction of natural incidence of the disease.

V. NEED FOR POLYVALENT PROTISTA VACCINE

In its 1987 annual Progress report PAU station made the following statement: "Request has been made for a visit of Dr. M. Ristic, University of Illinois, U.S.A., as a consultant to develop the area of Babesia immunodiagnostics and vaccines leading to the development of polyvalent vaccines and diagnostics (theileriosis, babesiosis and anaplasmosis) during, 1988. A research proposal to develop this area of work has been submitted separately as joint venture between PAU, Ludhiana and the University of Illinois (Dr. Ristic). We at PAU Regional Research Center have made significant progress on T. annulata piroplasms immunity." All other stations have also expressed a similar concern with Babesia and Anaplasma problems in India and also requested that a joint research arrangement be made between their respective station and the University of Illinois. Consequently, it is clearly recognized that preventive and control measures must be developed for all three diseases in a form of a polyvalent vaccine.

In order to accomplish this task the research program on theileriosis vaccine must be augmented in India. This can best be accomplished by closer collaboration between stations working on

theileriosis vaccine and by some additional training in immunochemistry and tissue culture techniques. It is now clear that there are two culture systems underway in India for propagation of T. annulata and the development of vaccines against theileriosis. Each of these systems has its own merit. In addition a new rapid and specific serodiagnostic test for detection of anti T. annulata antibodies has been developed by PAU. This test should be of special benefit to those working with lymphocyte culture systems so that they may be able to more specifically detect and measure immune responses in cattle inoculated with antigens derived from their cultures. Each station working on theileriosis vaccine must carefully examine the potential of its vaccine to induce an immunity to homologous and heterologous challenge. Once this has been determined the vaccines that showed the broadest protective activity should be selected for field tests in various regions of India.

#### VI. TRANSFER OF BABESIA - ANAPLASMA VACCINE TECHNOLOGY

For the purpose of making available for use in India a triple vaccine for immunization against theileriosis, babesiosis and anaplasmosis, an arrangement for an early transfer of Babesia and Anaplasma vaccine technology from the University of Illinois to the

subproject must be made. With reference to the transfer of vaccinal technology of culture derived immunogens of B. bovis and B. bigemina and the use of attenuated and inactivated A. marginale vaccines, only selected aspects can be offered through short-term training. The full transfer of these vaccine techniques for use under Indian field conditions, however, can be accomplished by the establishment of a specific joint research program. A proposal for such a transfer program has already been prepared by scientists from PAU. A realization of such a transfer program must proceed in three phases. During the first phase selected members of the PAU team will be trained at the University of Illinois in Babesia-Anaplasma cultural techniques, antigen production and vaccine formulation. In small vaccine trials they will receive detailed familiarization with vaccination techniques, measurement of immune response and challenge procedures. During the second phase similar experiments will be conducted under laboratory conditions in India. In the third and final phase of this program Babesia - Anaplasma vaccines along with the best Theileria vaccine must be examined under field conditions with complete epidemiologic monitoring of the results.

The accomplishment of this transfer program is estimated to cost approximately \$700,000 per year in direct costs. It will require a period of four to five years to accomplish the three phases of this new aspect

of the program. The initial stages of development of large scale vaccine production methods for the three organisms (Theilaria, Babesia, and Anaplasma) as a triple vaccine should also begin near the end of this five year period.

The University of Illinois holds patent rights on the above Babesia Anaplasma products. On one of the Babesia species, B. canis, the University has assigned the patent rights to Rhone Merieux of Lyon, France. Using this technology, the company has developed the first commercial blood protozoan vaccine. The vaccine is manufactured and marketed under the trade name "Pirodog". During a two-year period hundreds of thousands of dogs have been vaccinated with excellent results.

It is the consultants feeling that with the above contractual arrangement and in consideration of the economic needs of India as a developing country, the University of Illinois administration would be receptive to the release of patent rights and the transfer of vaccinal Bebesiu - Anaplasma technology for use in India only as part of an expanded collaborative research program between PAU, ICAR and the University of Illinois.

## VII. SUMMARY AND RECOMMENDATIONS

1. Short term training in the U.S.: The principal objective of the currently funded program is to provide additional specialized training for selected Indian scientists in order to ensure more efficient research toward the development of prophylactic and serodiagnostic methods for control of the three principle blood protistan diseases. Accordingly, those engaged in the development of theileriosis vaccine should be provided with additional training in immunochemical methods with emphasis on specific detection of antigenic material and quantitation of vaccinal doses. Further briefing will also be needed in the use of serodiagnostic methods for monitoring immune responses in vaccinated animals and field epidemiologic studies.

The investigators interested in tick-derived vaccine should be provided with additional knowledge regarding the development of the organism in the tick and the means of monitoring microbial pathways in this vector.

This training is of a specialized nature and will occur within a relatively short time period for each trainee. Because of the specialized nature of the training it will require considerable

coordination and preparation prior to the actual visit of scientists to Illinois.

2. Research on Theileriosis Vaccine: Two in vitro cultivation systems are currently used as a means of producing vaccinal T. annulata antigens. A more traditional method of cultivation of T. annulata is by selection of less virulent schizont population using high passage lymphocyte cultures. This methodology is being practiced at IVRI, NDDB, HAU, and PAU. A new cultivation method was also recently developed at PAU. In this method co-cultures of macrophages and lymphocytes initiated from the blood of infected animals was used to produce piroplasm forms of T. annulata that could be obtained from the supernatant culture media. This antigen was used to develop DOT-ELISA diagnostic method for detection and measurement of anti-T. annulata antibodies. In addition, in a very preliminary experiment piroplasm antigens were found to induce protective immunity to T. annulata sporozoite challenge.

The success at various levels with lymphocyte cultures and the above new cultivation method offer a strong promise that Indian scientists are under way of developing a vaccine against theileriosis. To that goal, the following specific studies must be made. Each group must clearly

define and characterise its vaccinal antigens. The antigenic relationship between lymphocytic and erythrocytic antigens must be investigated. The newly developed DOT-ELISA test must be used by all groups for monitoring immune responses in vaccinated and challenged animals and for seroepidemiologic studies. Finally it must be determined which specific vaccinal method offers the broadest protection not only against homologous but also against heterologous challenge. This is because T. annulata isolates from various sections of India may be antigenically different.

3. Transfer of Babesia-Anaplasma Vaccine Technology: The need for Babesia and Anaplasma vaccine along with that for Theileria has been clearly recognized by all stations. A polyvalent vaccine against the three agents appears as the only realistic way of controlling blood protista diseases in India. With the work on theileriosis vaccine well under way, the transfer of Babesia and Anaplasma vaccination methods from the University of Illinois to India is mandatory if an early solution of the problem is to be achieved. It is proposed that a new university of Illinois - Punjab Agricultural University collaborative program with ICAR-AID coordination be established to expedite the transfer of scientific information and expertise.

Based on the survey of potential scientific power, local interest for and economic input into the subproject and the available physical facilities, PAU appears to be the most appropriate station for a collaborative transfer of Babesia-Anaplasma vaccine technology. This transfer may be accomplished by way of a special contractual arrangement between the University of Illinois and the PAU through full understanding of the ICAR program coordinating Center (Dr. M. N. Malhotra in charge). The total monitoring of the transfer of Babesia and Anaplasma technology must also remain under the control of the coordinating center. Briefings on the progress of this technology transfer must be made available by periodic progress reports from the subproject coordinating center to the ICAR and AID.

For more specific information regarding the transfer of this technology please see Section VI of this report.

#### VIII. ADDENDUM

##### 1. Annual Workshop

Within the frame of the coordinating blood protista program the annual workshop was held at Haryana Agricultural University, Hissar, on

January 13 and 14, 1988. The opening address was that of the Vice-Chancellor of the HAU followed by Dr. Acharya of the ICAR. The consultant chaired the technical program of the workshop at which principal investigators of the five stations presented their progress reports. The representative of the new sixth station from Patna (Bihar) was also present but at this time no progress report was available.

With regard to theileriosis two stations seem to have made considerable research progress. Using a high passage infected lymphocyte cultures Dr. D.K. Singh of NDDB vaccinated with good results nearly 10,000 cattle including 1000 imported Holstein cattle. He commented, however, that some 20% of these cattle later developed bovine anaplasmosis indicating the need for a polyvalent vaccine. Dr. A.S. Grewal of PAU introduced a new in vitro system for production of T. annulata immunogen. He also reported obtaining blood phase piroplasms from infected erythrocytes which he then successfully used for the development of a DOT-ELISA serologic test. The test is a new important tool for future immunologic monitoring of experimental cattle as well as seroepidemiologic studies. Such studies are an important prerequisite to field vaccination trials.

HAU and IVRI also claimed good vaccination successes with their T. annulata vaccines, however their results were less conclusive.

All stations reported observing Babesia and Anaplasma occurring as mixed or single infections among cattle in their respective areas. Hence, they clearly indicated the need for a polyvalent vaccine.

Regarding the research for 1988 two principal recommendations have been made: (1) All potential theileriosis vaccines must be tested centrally at an independent station, (2) All seroepidemiologic studies of theileriosis will be conducted by use of the newly developed DOT-ELISA test system. The central testing of the vaccine will be done on 2-month old calves at PAU. The principal investigator of the respective station must provide the vaccine and challenge material to be used in the experiment at PAU. An official report of the test results will be submitted to Dr. Malhotra and the ICAR coordinating centers.

## 2. Training Program

As projected in a communication from ICAR to the AID and Winrock International, the five selected Indian participants are to start their training in the U.S. by April 1, 1988. It is anticipated that necessary

contractual arrangements for this training will be made prior to the above date.

### 3. Visit to PAU

During his three-day visit to Ludhiana the consultant had an opportunity to meet with the Vice-Chancellor and other officials of PAU, observe research and animal holding facilities and discuss the current and future research with members of the blood protista program.

The officials of the PAU have shown a great interest for the tick-borne disease program and indicated their strong support for it. The newly constructed tick-borne disease facilities are most impressive. Once accomplished and properly staffed this complex should become a major, world-wide leading institution for blood diseases research.

The principal investigator for the blood protista program, Dr. A.S. Grewal, is a most competent and knowledgeable scientist. With his leadership, the current staff and a research team to be recruited, the centre should be able to accomplish the task of developing a polyvalent vaccine against the three blood protistas.

The consultant had a lengthy discussion with Dr. Grewal and his team regarding their research needs and technical information. Some of this material will be forwarded to PAU in the very near future.

A joint program between the university of Illinois and PAU has been discussed with the Vice-Chancellor, other officials and researchers and has obtained their strong support. Important aspects regarding equipment, supplies and additional technical staff for this joint program has been elaborated upon and projected in writing.

In summary, PAU appears to be the most appropriate station for the expansion of the program into a collaborative arrangement aimed at the development of a triple vaccine for prevention of the three blood diseases in India.

P.S.: Liquid Nitrogen is essential for the operation of Blood Protista, Embryo Transfer, Artificial Insemination and Biophysical Sciences. Currently Liquid Nitrogen is being procured by long distance transportation and even then it is not readily available. A separate grant is needed to install a plant for the production of Liquid Nitrogen on the campus of the Punjab Agricultural University.