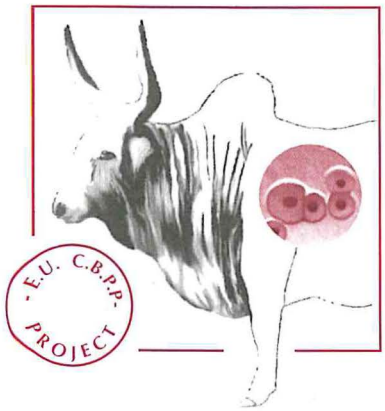

CBPP E/U PROJECT

ACCOUNT N° 5100.35.94.917 (5REG917)

ANNUAL SCIENTIFIC REPORT WORKPLAN N°2

May 1998
by J.J.TULASNE - CIRAD-EMVT- FRANCE
COORDINATOR OF THE PROJECT

CIRAD-EMVT, Campus Int'l de Baillarguet, B.P. 5035, 34032 Montpellier Cedex 1, France
Tel. : (33) 467 593 724 - Fax : (33) 467 593 798 - Mail : tulasne@cirad.fr

❖ *COMPONENT 1 : DEVELOPMENT OF A CBPP / ISCOM VACCINE*

❑ *UPPSALA UNIVERSITY (SWEDEN)*
B. MOREIN, I. ABUSUGRA.

- *ISCOMs induce a long lasting antibody mediated and cell mediated immune response **in mice and cattle**.*
- *On the other hand experimental CBPP ISCOM vaccines induce high **mucosal antibody responses in mice** immunized intranasally and subcutaneously.*
- *ISCOM seem to be an effective **delivery system** for the parenteral and mucosal routes of immunization.*

*See Sweden's report in **Appendix 1**.*

❑ *CVL, WINDHOEK (NAMIBIA)*
O. HÜBSCHLE, H. T.KAURA.

- ***Preliminary experiments have been achieved successfully to reproduce CBPP (intubation and contact animals).***
- ***A first ISCOM vaccine trial** is in progress in 1997 with 10 vaccinated and 10 control animals.*

*See Namibia's report in **Appendix 2.***

❑ *RENNES UNIVERSITY (FRANCE)*
H. WROBLEWSKI

- *Mycoplasmas induce inflammatory and humoral responses against their **membrane lipoproteins**.*
- *Attention should be paid to these lipoproteins to understand the **pathogenicity of mycoplasmas** and their interactions with the immune system, and on the other hand, to develop new vaccines and serodiagnostic techniques.*
- *MmmSC lipoproteins (p42, p65) **have been selected and purified**.*
- *A large scale purification method is in progress.*
- *Lipoproteins have been supplied to Uppsala to prepare experimental ISCOM vaccines.*

*See Rennes' report in **Appendix 3.***

❑ *IMMIS, MUNICH (GERMANY)*
G. WOLF

✿ *No report.*

❖ *COMPONENT 2 : IMPROVEMENT OF DIAGNOSTIC TECHNIQUES*

❑ *CIRAD-EMVT (FRANCE)*
F. THIAUCOURT

✿ ***Improvement of diagnostic techniques.***

** The final purpose of this study is to **validate both the currently used complement fixation test and the c-ELISA,** according to the real epidemiological situation of the herds, by comparing the sensitivity, specificity, intra laboratory reproductivity, userfriendliness and cost of both tests.*

** It is now considered **that all African laboratories of the network** (Cameroon, Ethiopia, Kenya, Namibia and Uganda) participating in this validation **are able to perform some serological analyses** as shown by the various reports.*

** However, the **primary goals of the validation above mentionned are not yet achieved**:*

*- To compare the real sensitivity and specificity of the 2 tests, it is absolutely necessary to **gather reliable epidemiological informations** on the sampled herds to determine the time elapsed between the contamination of the herd and the time of sampling, in order to observe a possible difference of capacity of detection between the 2 tests.*
***This point is difficult to achieve in the field**.*
*- Another goal is to check **if vaccination induces the production of antibodies detectable by c-ELISA.** Only Ethiopia could achieve such a study. **Apparently, antibodies after vaccination are detected by c-ELISA only for a short period, roughly 1 month**.*
If these preliminary findings are confirmed, it should be indicated to use the c-ELISA test to detect outbreaks in countries where vaccinations are performed.

** In conclusion of a recent collaboration between OVI (Onderstepoort, South Africa) and Cirad-emvt, it has been shown that **CFT and c-ELISA have similar sensitivities and a good correlation** has been established between the 2 laboratories.*

**** Reminder on training** :*

*- Cirad-emvt organized in Oct. 1996 in Montpellier (France) a **workshop on the validation of the CBPP c-ELISA test** for the African technicians of the network.*
*- In August 1997, Cirad-emvt participated in a **training course devoted to the CBPP c-ELISA test**, held under the umbrella of IAEA at the Onderstepoort Veterinary Institute (South Africa) for the Southern African technicians.*

** It is considered that the **different uses of the serological tests** for CBPP are mainly :*

- *To confirm the CBPP outbreaks*
- *To assess the prevalence of the disease in a country*
- *To assess the rate of covering in the frame of a vaccination campaign.*

*See CIRAD-EMVT report in **Appendix 4**.*

❑ *CONTRIBUTION OF AFRICAN LABORATORIES TO COMPONENT 2*

See in Appendices the country reports of :

- *LANAVET, Garoua (CAMEROUN) A. YAYA in **Appendix 5**.*
- *NVI, Debre Zeit (ETHIOPIA) LAIKE MARIAM YIGEZU and F. ROGER, in **Appendix 6**.*
- *KARI (KENYA). H.O. WESONGA in **Appendix 7**.*
- *CVL, Windhoek (NAMIBIA) H.T. KAURA in **Appendix 2**.*
- *LIRI (UGANDA) E. TWINAMASIKO in **Appendix 8**.*

❑ *CONTRIBUTION OF IZSAM (ITALY) TO COMPONENT 2*

*Italy proposes **modifications of the CFT technique** with reduced concentrations of red blood cells, hemolysin, and complement.*
There is a good concordance between the original and the modified CFT test.

*See Italy's report in **Appendix 9.***

❖ *COMPONENT 3 : IMMUNOPATHOLOGY - IMMUNOLOGY*

❑ *CIRAD-EMVT, FRANCE*
L. DEDIEU, V. BALCER

❑ *LANAVET, CAMEROUN*
J. LOPES DE LIMA

- *During Workplan 2, a second **experimental infection** was held in Garoua to verify the results previously obtained and to study the **correlation between immune response and protection**.*

- ***Experimental infection process in Cameroon is satisfactory.***

- *The MmmSC specific immune response of CBPP infected cattle is mainly based on **CD4 T cells**, the balance between the 2 **subpopulations (TH1 - TH2)** is not yet determined.*

- ***Interleukin specific** assays in progress will help and better characterize the immune response and understand the **immunopathology** of CBPP.*

- *On the other hand, research are in progress to select the **specific proteins** of MmmSC involved in the **immune reaction (mainly responsible for a long term protection).***

*See CIRAD-EMVT report in **Appendix 10**.*

*See Cameroun's report in **Appendix 11**.*

❑ *LNIV, Lisboa, PORTUGAL*
M.H. FERROHNA

- *Samples of lung lesion and lymph nodes from Cameroon (experimental infections) and Portugal (natural infections) have been collected for **histopathology and immunochemistry** (detection of mycoplasma, immunoglobins, cell populations), for a better undertanding of the CBPP **immunopathology.***

*See Portugal's report in **Appendix 12**.*

❑ *In parallel, **IZSAM (ITALY)** introduces some histological, immunohistochemical and ultrastructural studies.*

*See Italy's report in **Appendix 9.***

❖ *COMPONENT 4 : TRAININGS AND MEETINGS*

MEETINGS

- ❑ *Second annual meeting of the CBPP/EU Project in LANAVET, Garoua (Cameroun) in July 1996, with the representatives of all the project partners.*

TRAININGS

- ❑ *Workshop on the validation of the c-ELISA for CBPP in CIRAD-EMVT, Montpellier (France) in October 1996, with technicians of the 5 African laboratories.*
- ❑ *Dr YAYA, LANAVET, Garoua, Cameroun : c-ELISA training in October 1996*
- ❑ *Dr I. ABUSUGRA, Uppsala University, Sweden : cell mediated immunity training in IMMIS, Munich, Germany, in December 1996.*
- ❑ *Dr LOPEZ DE LIMA, LANAVET, Garoua, Cameroun : training in France in February 1997.*

MISSIONS

- ❑ *Dr L. DEDIEU, CIRAD-EMVT, Montpellier, France : visit to Berne, for a tentative collaboration with Berne Univeristy, Switzerland, for immunological studies on experimentallly infected animales., Sept. 1996.*
- ❑ *Dr J.J.TULASNE, CIRAD-EMVT, Montpellier, France: participation in the OAU/IBAR Congress on East Africa (Nairobi, Kenya) + visit to NVI, Debre Zeit for follw up of the CBPP/EU experiemnts . March 1997.*
- ❑ *Dr J.J. TULASNE, CIRAD-EMVT, Montpellier, France : participation in the 12th Conference of the O.I.E. Regional Commission for Africa, Pretoria (South Africa), in January 1997, and extension for a visit in CVL, Windhoek (Namibia), for the follow-up of the project and implementation of the ISCOM vaccine trial in North Namibia.*
- ❑ *Mrs V. BALCER, Dr L. DEDIEU, CIRAD-EMVT, Montpellier, France : follow-up of the experiments inLANAVET, Cameroon. Blood sampling + bronchoalveolar lavages, samples transport in liquid azote. May 1997.*
- ❑ *Dr J. LOPEZ DE LIMA, CIRAD-EMVT, in post in LANAVET, Cameroon : transport of endoscopic equipment to France for repair + transport of frozen samples to CIRAD-EMVT . Oct. 1997.*
- ❑ *Dr F.THIAUCOURT, CIRAD-EMVT, Montpellier, France : participation in an IAEA CBPP formation, OVI, Johannesburg (South Africa) + visit to Windhoek, for implementation of the T1SR-T1/44 experiments. June 1997.*
- ❑ *Dr A. FILOMENA DE JESUS, LNIV, Portugal : collection of samples from LANAVET, Cameroon for immuno-histo chemical analyses in LNIV., sept. 1997.*

❖ *SOME PUBLICATIONS IN 1996 / 1997*

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DEDIEU L., BREARD A, LE GOFF C., LEFEVRE P.C. (1996) - Diagnostic de la péripneumonie contagieuse bovine (PPCB) problèmes et nouveaux développements. Revue Sc. et Technique de l'OIE, 15 (4).In press

JAN G., BRENNER C., WROBLEWSKI H. (1996) - Purification of Mycoplasma gallisepticum membrane proteins p.52 p67 (pMGA) and p77 by high-performance liquid chromatography. Protein Express.Purif. 7:160-166.

JAN G., FONTENELLE C., VERRIER F., LE HENAFF M., WROBLEWSKI H. (1996) - Selective acylation of plasma membrane proteins of Mycoplasma mycoides subsp. mycoides SC, the contagious bovine pleuropneumonia agent. Current Microb., 32, 38-42.

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THIAUCOURT F., LORENZON S., DAVID A. (1997) - Application d'outils de biologie moléculaire à certaines mycoplasmoses des ruminants. Cahiers Agricultures 6: 145-148

TULASNE J.J., LEFEVRE P.C., BLANCOU J. (1996) - Experience with veterinary vaccines in warm climates. In : new approaches to stabilization of vaccines potency (F. BROWN Ed.), Dev. Biol. Stand. Basel, Karger. Vol 87, 33-42.

TULASNE J.J. (1997) - Actual status and trends of vaccination against CBPP in Africa. Presentation during the 12th Conference of the O.I.E. Regional Commission for Africa, Pretoria (South Africa).

TULASNE J.J., LITAMOI J.K., MOREIN B., DEDIEU L., PALYA V.J., YAMI M., ABUSUGRA I., SYLLA D., BENSAID A. (1996) - Contagious bovine pleuropneumonia vaccines : current situation and needs for improvements. Revue Sc. et Technique de l'OIE, 15 (4).In press.

APPENDIX 1

UPPSALA UNIVERSITY
SWEDEN

SCIENTIFIC REPORT

Pr B. MOREIN
Dr I. ABUSUGRA

SWEDISH UNIVERSITY OF AGRICULTURAL SCIENCES
SLU

Department of Veterinary Microbiology
Section of Virology

Uppsala Anual Report, November, 1997

In the previous report we have shown that a large number of Mmm antigens are incorporated into iscoms. These iscoms are very immunogenic inducing long lasting antibody mediated (AMI) and cell mediated (CMI) immune responses in both mice and cattle. These results are included in the first publication which is now in press (enclosed). Some sera were sent to Namibia for complement fixation (CF) testing.

The subsequent studies analyses the mucosal immune response in mice after intranasal (i.n.) and subcutaneous (s.c.) immunizations resulting in the second publication which is now being submitted. Serum and lung secretion antibody responses have been studied.

From this study it was evident that the iscom is an effective delivery system for both parentral and mucosal modes of immunization. After i.n. immunization prominant antibody responses are achieved in both serum and in lung secretion. In the latter the IgA response is strong.

Three to five fold more antigens were recognized by serum antibodies in W.B. than by lung secretion antibodies. A likely explanation for that is that the concentration of immunoglobulins is about 100 to 300 fold higher in serum than in the secretion. Serum from mice immunized i.n. detected more bands than serum from mice immunized s.c. After i.n. immunization different migration patterns were detected in secretion respectively in serum compared to that after s.c. immunization. The many fluffy bands detected in serum and also in secretion after i.n immunization indicate antibody reactions with carbohydrate structures which suggests an increased ability of carbohydrate antigens to induce antibody response via the mucosal mode of immunization as shown by Carol et al. (1997).

Postal address
Biomedical Centre, Box 585
S-751 23 UPPSALA
SWEDEN

Visiting address
Husargatan 3
Bldg A9:2

Tel.
+46 (0)18-17 45 71 (secr)
+46 (0)18-17 40 00 (exchange)

Fax
+46 (0)18-50 46 03

The third manuscript, which is in progress, concerns studies investigating membrane antigens containing carbohydrate structures. The aim is to find out whether the carbohydrate structures do induce immune response or not.
Antigens treated with Proteinase-K, were analysed by W.B. using sera from mice immunized with Mmm iscoms, showed that several bands were not detected after proteinase-K treatment, while some bands were still detected by the antibodes. This would indicate that some carbohydrates are connected with proteins and some not. Furthermore, it seems that antibody responses can be induced by iscoms to carbohydrates which are not tied to proteins presumably glycolipids, but possibly also to carbohydrates tied to proteins. In general these treatments indicate that the antigenic carbohydrates were recovered in aqueouss phases where also proteins were recovered. Studies are carried out to confirm whether the protein-carbohydrate associations are glycoproteins.

Iscom vaccine was sent to Namibia and is now being tested in a controlled field vaccination experiments by Dr Otto Hübschle and Dr Harold Kaura. In this experiment 10 animals have been vaccinated with iscom and 10 animals are left as non-vaccinated controls. These animals are exposed to animals proved to be infected with a recent low passage virulent Mmm isolate. The animals are now followed clinically.

Professor Bror Morein

Dr Izzeldin Abusugra

Uppsala, November the 6th 1997

ABSTRACT

The purpose of this study was to explore the iscom as a mucosal delivery system for CBPP antigens. Balb/C female mice were immunized intranasally (i.n.) and subcutaneously (s.c.) twice, 7 weeks apart with three different doses (3, 10 and 20 µg) of mycoplasma mycoides antigens incorporated into an iscom experimental vaccine. Two weeks after the second i.n. immunization the antibody responses in secretion extracted from the lungs were tested by ELISA for total IgG, IgA and in W.B. Prominant serum antibody responses were detected after the first immunization and those were strongly boosted by the second dose both after the intranasal (i.n.) and the subcutaneous (s.c.) immunizations in a dose dependent manner. High levels of both IgG1 and IgG2a and IgG2b serum responses but only low levels of IgG3 titre were obtained after both i.n. and s.c. modes of immunization. In lung secretion high IgA titres were obtained in a dose dependent manner. Also the IgG1 and IgG2a responses were high in lung secretion while the IgG3 response was low.

Lung secretion detected only about 9 bands in Western Blott both after i.n. and s.c. immunizations. In contrast a high number of bands (> 30) were detected by serum antibodies both after i.n. and s.c. immunizations. Interestingly after i.n. immunization different antigens and antigenic determinants were detected in W.B. both by lung secretion and by serum reflected by different W.B. pattern than after s.c. immunization.

INTRODUCTION

. At the end of the 18th century contagious bovine pleuropneumonia (CBPP) could be distinguished from other respiratory diseases as being caused by mycoplasma mycoides subsp mycoides-SC. However, evidence based on clinical, epidemiological and pathological information revealed a long history of the disease. Hudson (1971) reported that the disease originated in Europe and was dissiminated by trading to other countries.The disease is highly contageous and responsible for great economical losses in cattle. It is still endemic in Africa and some Asian countries while control measures eradicated the disease from most of Europe at the end of 19th century and later also from US and Australia.

Vaccines have been used for a long time. In Africa the Willem's method for s.c. vaccination (Provost,1987) is most prevalent in spite of the fact it leads to inflamatory reactions and even mortality. Inactivated vaccine strains have been tested with varying results but not in the field (Pearson and lloyd, 1971, Gray et al, 1986). The vaccines in use in Africa are KH3J and T1 (Brown et al, 1965; Hudson, 1964a; Karst, 1971). They sometimes may potentiate the disease (Provost et al, 1987) in addition they have a relative short duration of post-vaccinal immunity (Msiga and Domenech, 1995). When these vaccine strains were adapted to grow in streptomycine containing media variants were obtained designated KH3J-SR and T1-SR. The T1/44 and T1-SR had their drawbacks (Provost, 1978) and the KH3J and its variants are no longer used. Thus, new vaccine strategies are required, and the mucosal respiratory route of immunization need to be explored to evoke the local immune defence. Recent development of adjuvants based on different properties e.g. targeting antigen presenting cells (APC), modulation of immune response and the physical presentation of antigen opens ways to explore new formulations of vaccines (Morein et al, 1996). The iscom is a delivery particle for vaccine use supplying a multimeric presentation of antigens with abuilt-in adjuvant which in the present study was explored for mucosal administration of CBPP antigens to the respiratory tract of mice.

Keywords: CBPP, Mmm, ISCOM, i.n., s.c., mucosal immunity, total serum antibody response, IgA lungs, IgA serum, IgG subclasses, Western Blots.

Abbreviations: Mmm; Mycoplasma mycoides subsp mycoides Small Colony type, CBPP; Contagious bovine pleuropneumomia, MEGA-10; n-decanoyl-methyl glucamine, Quil A; Spicoside Quil A (Quilaia saponaria molina), DAB; 3,3-diaminobenzidine, HRP; Horseradish peroxidase, TMB; Tetramethylbenzidine, H_2O2_2; Hydrogen peroxide, PBS; phosphate buffer saline, T; Tween-20, PBS-T; phosphate buffer saline-Tween, SDS-PAGE; SDS-polyacrylamide gel electrophoresis, NC-paper; nitrocellulose filter paper, WB; Western blots, SD; Standard deviation , SEM; Standard error meam, r.t. Room temperature.

Corresponding author. Tel : +46-18-471 40 74; Fax: +46-18-504603; E-mail: Izzeldin.Abusugra@bmc.uu.se

MATERIALS AND METHODS

Chemicals and solutions

PBS Buffer. Methophane (Methoxyflurane).
Quil A (Escotec, Stockholm, Sweden). Prepare 20% solution in distilled water. Devide into aliquotes and save at –20°.
Heparin 5000 IU/ml prepare 0.1% and 1% solutions in PBS.
The detergent MEGA-10 was obtained from Bachem, Bubendort, Switzerland.
The lipids, cholesterol and 1-3-Phosphatidyl choline from egg yolk (Both grade 99%-100%), The substrate DAB were from Sigma (St. Louis, Mo., USA).
Rabbit anti-mouse immunoglobulins labeled with HRP were from Dakopatts (Copenhagen, Denmark).
TMB reagent, H_2O2_2 and coating buffer tablets were purchased from Svanova (Uppsala, Sweden).
Tween-20 was from Merk (Darmstadt, Germany).

Mycoplasma

Mmm strain Afade was supplied by Pathotrop laboratory, CIRAD-EMVT, Montpellier, France.

Formation of iscoms

Whole Mmm Cell iscoms were prepared according to the method of Morein et al. (1984) modified by Lövgren et al. (1987). Briefly, to mycoplasma sample the detergent MEGA-10 was added to a final concentration of 2% and left for 2 h at room temperature. The solubilized material was layered on top of 10% sucrose containing 0.5% MEGA-10 with a botom layer of 30% sucrose. The solubilized proteins were seperated from insoluble material by centrifugation at 40.000 rpm as described before (Abusugra et al., in press). The top layer was collected, and cholesterol, phosphtidylcholine and Quil A were added and mixed in a proprtion w/w of 10:2:1:20 and extensively dialysed against PBS overnight at room temperature and for further 48 h in a cold room. The formed iscoms were further purified by sedimentation through a double layer of 10% and 20% sucrose.The sedimented iscoms were resuspended in PBS and kept at -70°C until use.

I.n. immunization of mice

Mmm iscoms in volume of 15 µl were administered i.n. in mice under Methofane anaethesia.

28 Balb/c female mice 8 weeks old were divided into 4 groups each of 7 animals. The mice in group 1, 2 and 3 received respectively 3, 10 and 20 µg iscom vaccine i.n. twice 7 weeks apart. Group 4 served as non-immunized control animals.
An additional group of 7 mice were immunized s.c. twice as for i.n. immunization.
The animals were bled at 2, 4, and 7 weeks after the initial dose and 2 weeks later after the boost another bleeding was taken.

Extraction of lung secretion

The secretory antibodies were extracted basically as described by Charlotta et al. (1995). I.n. and s.c. immunized mice were bled prior to death. Briefly, mice were injected intraperitoneally with 0.1 ml Of 1% heparin-PBS under anaesthesia. Mice were exanguinated and perfused with 20 ml 0.1% heparin-PBS into the right chamber of the heart and through the circulation system.The lungs were removed and trimmed. Each lung was kept in 3 mls 0.1% heparin-PBS and weights were recorded. The organs were washed with PBS and stored at -20 C for overnight. The saponin Quil A was added at a ratio of 1µl/mg of organ (to each 100 mg organ 100 µl of 2% saponin Quil A-PBS were added). After overnight incubation at 4°C, the lungs were sedimented at 13000 rpm for 10 minutes in Eppendorf 5415C centrifuge and supernatants were collected for antibody analysis.

Total antibody responses

Antibody titres in the sera of immunized animals were determined by ELISA as described by Voller et al. (1978). Briefly, ELISA plates (Nunc, Copenhagen, Denmark) were coated with whole mycoplasma cells of the strain Afade at a concentration of 2 µg/ml in coating buffer (50 mM carbonate buffer pH 9.6) and incubated overnight at 4°C. All washings were made with phosphate-buffered saline (150 mM, pH 7.5) containing 0.2% Tween-20 (PBS-T). The plates were blocked with 2% fat-free condensed milk powder in PBS-T (blocking buffer) for 1 h at 37°C. Antisera were 3-fold diluted in blocking buffer and incubated for 1 h at 37°C. HRP conjugated rabbit anti-mouse were diluted 1:10.000 in blocking buffer and incubated for 1 h at 37°C. The enzyme reaction was visualized by addition of substrate buffer (TMB, H_2O2_2) in PBS. After 10 to 15 min the reaction was stopped by 2 M H_2SO_4 and the absorbance was measured at 450 nm with a Titertek Multiscan spectrophotometer (Flow Laboratories, Irvine, Scotland). The sera from unvaccinated mice were used for calculating cut-off. The cut-off was determined as the mean OD value of the negative reference

serum plus 2 SD and the antibody titre (end point) is expressed as the last dilution giving an OD value higher than the cut-off as described before (Abusugra et al. 1997 (in press).

IgG subclass responses

IgG subclasses in lung extract and serum of mice immunized i.n. or s.c. were measured by ELISA. ELISA plates coated with 2µg/ml whole mycoplasma were washed three times with PBS-T. Lung extract or sera in blocking buffer were 3-fold diluted. Specific goat anti-mouse IgG1, IgG2a, IgG2b and IgG3 (Nordic immunology) and HRP-conjugated rabbit anti-goat immunoglobulin (Dakopatts, Denmark) were used. All washings were made for three times and all incubations were at 37°C for 1 h under slight agitation. The enzyme reaction was visualized by addition of TMB, H_2O2_2 buffer the reaction and stopped 10 to 15 minutes later by 2M H_2SO_4 the absorbance was measured and the titre was determined as described before (Abusugra et al, in press).

ELISA for IgA in blood and in lung extract after i.n. or s.c. immunization

High binding ELISA plates Nunc, Denmark) were coated with 100 µl of Mmm at a concentration of 0.4 µg/well in the coating buffer (50 mM carbonate buffer pH 9.6) and incubated at +4°C for overnight or more. All washings (x3) were made with PBS-T. The plates were blocked with 200 µl of 2% BSA in PBS-T for 1h at r.t. under constant shaking. 100 µl of lungs extracts or serum in blocking buffer were 2-fold diluted and incubated at r.t. for overnight. 100 µl of 1:10.000 dilutions of Biotinylated Goat-anti-Mouse IgA in the blocking buffer were added and incubated for 1 h at r.t. under constant shaking followed by addition of 100 µl of 1:10.000 dilutions of HRP-Strept-avidin conjugate in blocking buffer and incubated under the same condition. The enzyme reaction was visualized by addition of 200 µl substrate buffer (TMB, H_2O_2) for 15-20 minutes at r.t. after which the enzymatic reaction was stopped by 50 µl of 2M H_2SO_4 and the absorbance was measured at 450 nm with a Titertek Multiscan spectrophotometer. Lungs suspensions and sera of control animals were used as background for calculating the cut-off as described before (Abusugra et. al. 1997, in press).

Electrotransfer of Mmm antigens for Western blots

Western Blot was made according to Towbin et al, (1979). Mmm iscoms were separated in an 12% SDS-PAGE after which they were transferred to a nitrocellulose membrane filter from Schleicher and Schüll, Germany. The membrane was blocked by soaking in blocking buffer (PBS-T, pH 7.2

containing 2% fat-free condensed milk) under constant shaking. For details see Abusugra et al (in press).

RESULTS

Groups of 7 mice were immunized i.n. twice 8 weeks apart with 3, 10 or 20 µg of Mmm membrane proteins incorporated into iscoms. One group of mice were immunized twice s.c. 8 weeks apart with 3 µg of Mmm iscoms.

Antibody responses in serum

The total serum antibody response of mice immunized i.n were measured by ELISA. A dose dependent rise was obtained through week 4 with a slight decrease observed at week 7. Two weeks after the boost immunization (week 8) a 100 to 1000-fold increase of the serum antibody levels were measured. The antibody response increased with increasing doses after the second i.n. immunization reaching serum titres of about $10^{4.5}$, $10^{5.5}$, 10^{6} respectively. (Fig 1). The s.c. immunization of mice with 3 µg iscoms induced serum antibody titres of about the same level of serum antibody response as 10 µg iscoms administered i.n.

IgG subclass responses in serum

High levels of the IgG1, IgG2a and IgG2b of serum antibody responses to Mmm were detected by ELISA both after 10 µg i.n. and 3 µg s.c. modes of administration with titres ranging between 10^5 to 10^6. The IgG3 responses were about a 100-fold lower than those of the other IgG subclasses both in serum and in lung extract (Fig 2a).

IgA responses in serum

The IgA response in the pooled sera increased in a dose dependent manner after two i.n. immmunizations of mice. The highest dose (20 µg) induced IgA ELISA titres of more than 1:8000 (Fig 3b).

Antibody response in lung secretion

IgG subclasses in lung extract

The Mmm specific IgG1 response was a four-fold lower in lung extract after i.n. immunization than after s.c. immunization. The latter reached titres of about 1:8000. The IgG2a response was of similar magnitude after both modes of immunization. The IgG3 titres were low (Fig 2b).

IgA response in lung extract

The IgA responses in the pooled lung extract increased in a dose dependent manner after two i.n. immmunizations of mice. The highest dose (20 µg) induced IgA ELISA titre more than 1:8000 XXXX segnificanceXXX (Fig 3a).

Antigen of Mmm incorporated in iscoms reacting with lung extract or serum from mice immunized i.n. or s.c.

The proteins of Mmm were resolved in 12% SDS-PAGE and transferred to a NC-paper. Lung extracts (Fig 4A) from mice immunized twice i.n. reacted with 7 to 9 bands, of which 3 are distinct (Lane 2). Lung extracts from mice immunized twice s.c. detected about the similar number of bands as after i.n. immunization, 5 of which are distinct (Lane 3). The migration patterns of proteins detected by W.B. in lung secretion differed after i.n. and s.c. mode of immunization. At least 3 of the major protein bands of both showed different migration patterns. The rest of the bands are faint and most of them showed different migration patterns.

In Fig 4B sera from the same mice immunized i.n. (Lane 2) detected about 37 bands which in general are more fluffy than those detected after s.c. immunization. Only 3 of these bands are distinct. S.c. immunized mice detected about 27 bands of which 8 to 9 are very promenant (Lane 3).

DISCUSSION

The prominant immunogenecity associated with iscoms is partly related to effecient presentation of the antigens by antigen presenting cells (APC) resulting in prominant primary and secondary B and T cell responses (Höglund et al, 1989; I. Claassen, A. Osterhaus, 1992; Morein 1995). The iscom also exhibits its adjuvant activity by modulating T-helper cells towards Th1 type of response with IL-2 and IFN-γ production, but also the Th2 cytokine IL-4 is in some cases enhanced. I.e. a mixed type of Th1 and Th2 can be obtained (Villacres X; Morein 1996; Heeney Jonathan, 1997). Further, the T-cell response encompasses cytotoxic lymphocytes (CTL) both after parentral and mucosal modes of administration (for references see Morein et al, 1995).

Important for the development of new improved microbial vaccines with enhanced immunogenicity is the search for effective and safe adjuvant delivery system for induction of both systemic and mucosal immune responses (X). In the present study we achieved that goal in mice by i.n. immunization with Mmm antigens after incorporation into iscoms. A prominant antibody response was induced in serum after the first i.n. immunization (Fig 1) which was highly increased after the boost to surprisingly high level being of similar magnitude as that induced by s.c. immunization. Eventhough, the dose of 10 µg used for the i.n. immunization was higher than that used for the s.c. one it was not an exagerated dose as often reported for mucosal experiments. It should also be borne in mind that the technique for i.n. administration has not been optimized and perhaps only 5 to 20% of the dose might be properly distributed to the appropriate mucosal surface (X). In the Mmm iscom a large number of antigens, which probably are membrane bound, are incorporated. It seems that many or most of those induce high immune response after the i.n. administration as detected in serum by Western blot (see Abusugra et al., 1997, in press).

Mice immunized twice i.n. or s.c. responded with high levels of antibodies in serum distributed in the various IgG subclasses, of which IgG1 idicating a Th2 response and IgG2a and IgG2b indicating a Th1 response, were prominant. IgG3 response was low as mostly is the case. Also in lung extract after both modes of immunization high levels of IgG1 and IgG2a subclass responses were recorded. As for serum the IgG3 response detected was low. It is interesting to note that both high levels of IgG2a and IgA is produced after i.n. administration since the former is expected to be guided by the Th1 type of response and IgA

is more dependent on the Th2 type of response (X). From protection point of view it might be benificial that those responses are not counter acting each other(Immunology today X). As expected the systemic administration was not an effecient mode of immunization for induction of local mucosal immunity (Lazzel et al, 1984; Bergmann et al,1986). Thus, the current study emphasised the potential of the iscom as delivery system that promotes both systemic and local immune responses after i.n. immunization.

It is well recognized that it is difficult to evoke mucosal immunity with nonreplicating antigens, the striking exception being adjuvants formulations using cholera toxin (CT) or thermolabile toxins of E-coli (LT) (X) . However, both CT and LT are toxic in their unmodified form, but deletions of certain regions of the A subunit seem promissing in the sense that toxicity is reduced but the mucosal targeting and adjuvant activities are retained (X). There are now accumulating evidences that the iscom is an effecient non-replicating delivery system which induce secretory IgA, CTL and systemic immune responses (Morein et al; Lövgren et al; Mowat and Maloy, 1994). Neutralizing antibody has been considered the gold standard in measuring antiviral activity in vitro in studies involving poliovirus as well as other human viruses (Ogra et al; Cooper et al, X). Recently, it was also shown that iscoms loaded with respiratory syncytial virus (RSV) envelope antigens after i.n. administration induced virus neutralizing antibodies (submitted) detected both in serum and in lung secretion. Iscoms bearing the B subunit of CT induced high IgA antibody titres in the respiratory tract (submitted).

Western Blotting was applied to determine the reaction of antibodies in serum and lung secretion with Mmm antigens incorporated into iscoms. Lung extract detected, after both i.n. and s.c. modes of immunization, about the same number of bands but differences were observed both with regard to the migration patterns and to the intensity of bands showing similar migrations between the two modes of immunization. The number of bands detected by nasal secretion was much lower than that detected by serum. A likely explanation is that the total Ig in the sample of lung extract applied on the gel was low , about 1.5 µg while corresponding amount of the serum sample was high i.e. 500 µg total Ig probably also implicating that there is also a higher concentration of specific antibodies in serum (4A).

Serum tested after i.n. immunization detected a higher number of fluffy bands than after s.c. immunization (4B). This could indicate that the mucosal route

could recognize more antigens and antigenic determinants than systemic route (e.g. some proteolytic degradation of antigens may occur on the mucosal surface). The fluffy bands may indicate the presense of carbohydrate structures containing proteins or lipids, glycoproteins or glycolipids respectively. However, generally it is conceived that glycoproteins in contrast to glycolipids are unusual or nonexisting in bacteria (X). Apparantly, the different pathways of immunization recognized partly different repartoirs of antigens and consequently also different antibody responses reflected in W.B. after developement with serum and secretions. Some bands in W.B. were present after both modes of immunization while others were absent after one mode but present after the other mode of immunization and vice versa. In this context it is interesting to note that Carol et al, (1997) found that serum antibody responses to carbohydrate epitopes were more effeciently induced by i.n. immunization with iscoms loaded with echinococcus granulosus antigens than by s.c. immunization. The high number of bands detected in serum after i.n. immunization can be due to various factors as stated but for serum by Rhalem et al, (1993) i.e. some epitopes may be shared between different molecules e.g. oligosaccharidic side chains which might be better recognized after i.n. immunization. Further, some epitopes may be borne by molecules which are precursors or hydrolysis of other proteins or carbohydrates. In the previous study carbohydrate structures were detected in Lectin Blots (Abusugra et al, 1997, in press).

In conclusion, the iscom seems to be an effecient delivery system for both parentral and mucosal modes of immunization. The ensuing immune response after i.n. immunization is prominant both in serum and in the local lung secretion. Since the antigen recognition differs between the two modes of immunization one or the other mode may better recognize protective antigens or epitopes, which is an important aspect in the construction of protective vaccines.

APPENDIX 2

CVL - WINDHOEK
NAMIBIA

SCIENTIFIC REPORT

Dr O. HÜBSCHLE
Dr H.T.KAURA

Report Workplan 2

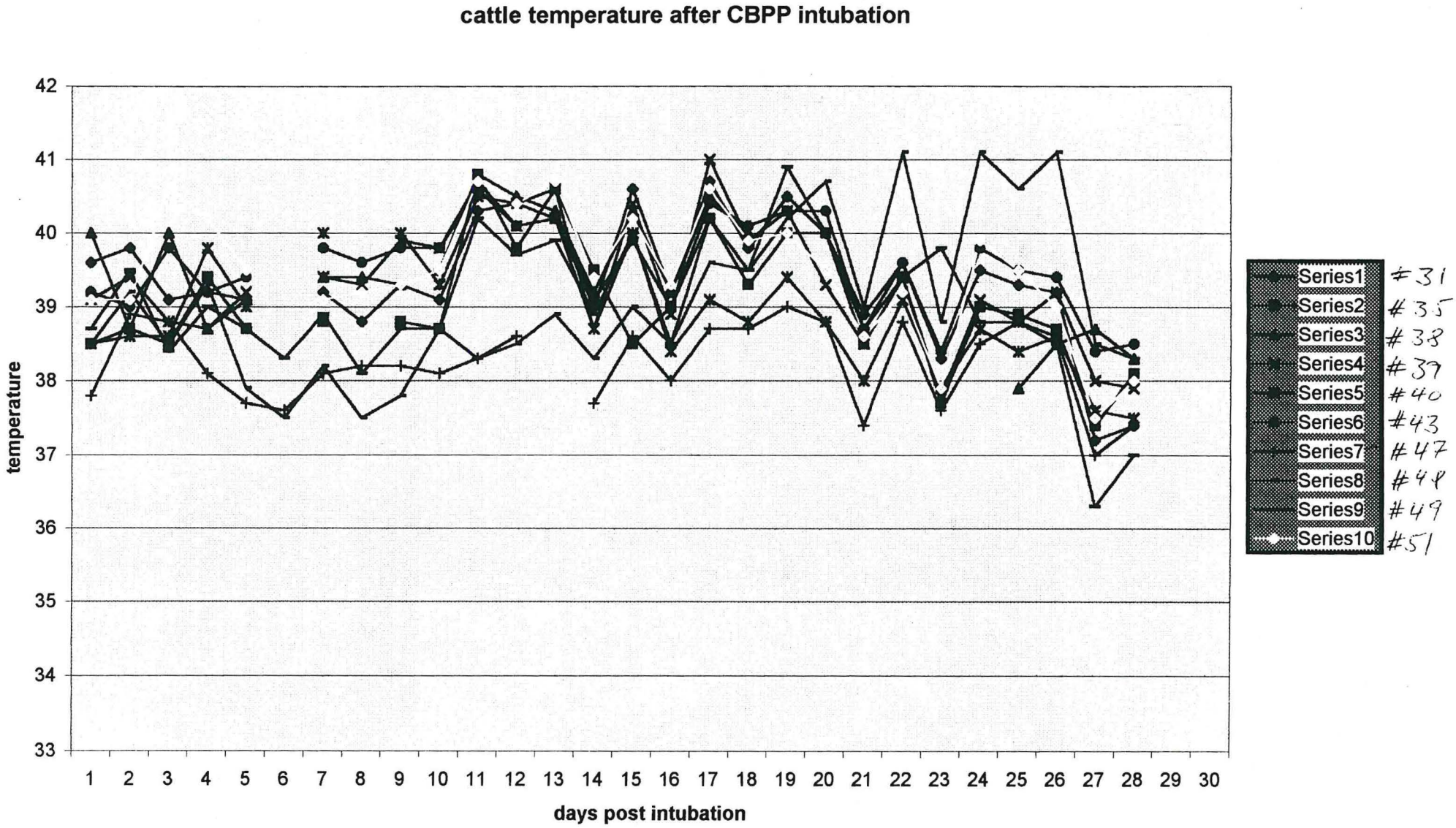
Central Veterinary Laboratory Windhoek Namibia

Component 1

Due to funding delay the field trial with the ISCOM-CBPP vaccine could only be started towards the end of May this year, as two months passed due to purchasing and testing the animals. All animals used in the ISCOM trial have been purchased from areas South of the Veterinary Cordon Fence and have thus never been in contact with CBPP. The first vaccination was done on the 16th of July1997 and the second vaccination was followed on the 4th of September 1997. As the authorities had started early in August with a vaccination campaign using T1/44 CBPP vaccine it has become difficult to find animals with full-blown CBPP. Therefore an initial experiment was conducted during August to reproduce experimentally CBPP in 3 animals and three contact animals kept with the intubated ones. As these initial experiments proved very successful using a recently isolated Mycoplasma mycoides strain, 10 animals of the fifteen used as a challenge source for the vaccinated animals were infected on the 23rd and 24th of September via intratracheal intubation. The construction of a mouth-restrainer by the farm manager of the experimental farm of the CVL thereby facilitated the operation considerably. As tube a horse stomach tube proved most suitable without causing any undue harm to the animals. Using this technique all ten animals could be intubated within 1 hour without any problems. Two weeks post infection, a marked temperature reaction was seen in the ten animals and substantial complement fixation titres were observed two weeks onwards post infection confirming that all animals have been successfully infected judged by these two criteria. The ten animals together with the five contact animals where therefore integrated into the original test herd of 30 (15 vaccinated - and 15 control animals) on the 23rd of October. Temperature is taken daily on all 45 animals and blood for serum is withdrawn weekly since the integration of all animals into one herd. The animals are kept inside a water-kraal during the night in order to mimic the natural situation as seen in the communal

farming practice (the kraal ensures thus a relatively close contact between infected animals and test animals.)

It is expected that the first control animals shall come down with CBPP towards the end of November when colleagues from Teramo will join the local project partners in examining the animals and hopefully be in a position to perform some post-mortem examinations .


cattle temperature after CBPP intubation
temperature
42
41
40
39
38
37
36
35
34
33
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30
days post intubation
Series1 #31
Series2 #35
Series3 #38
Series4 #39
Series5 #40
Series6 #43
Series7 #47
Series8 #48
Series9 #49
Series10 #51

Complement fixation titres of animals infected intratrachally with local Namibian Mycoplasma mycoides var. mycoides sc. strain

animal number	*cf.titer 22/07/97*	*cf.titer 03/10/97*	*cf.titer 08/10/97*	*cf.titer 15/10/97*	*cf.titer 22/10/97*
31	*neg.*	*a/c*	*> 1:320*	*1:320 ++++*	*1:320 ++++*
32	*neg.*	*1:160 +*	*> 1:320*	*1:320 +++*	*1:320 ++++*
33	*neg.*	*neg.*	*neg.*	*neg.*	*neg.*
35	*neg.*	*1:40 +*	*>1:320*	*1: 320 +++*	*1:320 +++*
38	*neg.*	*neg.*	*a/c*	*1:320 ++++*	*1:320 +++*
39	*neg.*	*1:20 +*	*1:320 +*	*1:320 ++++*	*1:320 +++*
40	*neg.*	*neg.*	*1:320 +*	*1:320 ++++*	*1:320 ++++*
41	*neg.*	*neg.*	*neg.*	*neg.*	*neg.*
42	*neg.*	*neg.*	*neg.*	*neg.*	*1:160 ++*
43	*neg.*	*neg.*	*1:320 +*	*1:320 +++*	*1:320 ++++*
47	*neg.*	*1: 160 +*	*1:320 +*	*1:160 ++++*	*1: 320 +*
48	*neg.*	*1:40 +*	*1:320 +*	*1:160 +*	*1:320 +++*
49	*neg.*	*1:40 ++++*	*1:320 ++*	*1:320 ++++*	*1:320 ++++*
51	*1:10 +*	*1:80 +*	*1:320 ++*	*1:320 ++++*	*1:320 ++++*

The following animals were intubated on two consecutive days [23/9and 24/9/1997] 31, 35, 38, 39, 40, 43, 47, 48, 49 and 51

Animal 32 was intubated on the 20th of August 1997 (pre-trial intubation)

Animals 41/ 42/ 33 were included in pre-trial intubation test as contact animals

Component 2

The PCR using CIRAD-EMVT primers continued to be used for routine diagnostic procedures and gave consistent results when compared to other identification techniques (i.e. isolation procedures). After some problems which have in the meantime been resolved both the complement fixation-test as well as the competetive-ELISA are now used on a routine basis. The serology section is testing all sera, which originate from known positive cases (post mortem results and if possible positive cultivation) to evaluate the c-ELISA test further on its suitability in large scale serological surveys.

In collaboration with Dr. T.Pearson, University of Victoria Canada, monoclonal antibodies against membranes of the Afade type strain have been prepared in Victoria/Canada. 35 supernatants were found to be positive tested in indirect ELISA on the immunising antigen and were found to be negative for irrelevant antigens, incl. human transferrin and T.brucei proteins.
All 35 supernatants were tested for species specificity as well as mab 117/5 (France) in indirect ELISA. Whole organisms from *Mycoplasma mycoides subsp. mycoides* (LC), YG-type strain, *Mycoplasma mycoides subsp. capri*, PG_3-type strain, *Mycoplasma capricolum*, California Kid-type strain and Mycoplasma bovine serogroup 7, PG_{50}-type-strain as well as T1SR-type strain.
Mab 117/5 is very strong reacting and SC-specific, 9 mabs out of the 35 supernatants are SC-specific, 5 out of these nine belong to a weak reacting group, 4 other mabs have higher titres, but the background increases with the time.
The strongest reacting mab is positive for SC- and LC mycoplasmas, a result that could be confirmed as well by immunoblot experiments.

The nine SC-specific mabs were used to test their reactivity and specificity towards SC and LC strains by immunoblotting and to choose possible candidates to isolate a clearly defined antigen.

3 out of the 9 mabs were SC-specific (7, 16, 5) , but their immune sera gave patterns in the same order like the protein pattern. All other 6 mabs were crossreacting with LC-strains.

The only candidate (mab 28) which showed a strong response towards a defined protein band, reacted with SC-strains and with a protein of LC-strains in the same molecular weight range, a result which could be demonstrated as well by tryptic digestion and by Triton X-114 separation.

The corresponding protein of SC-strains has been isolated by preparative electrophoresis.

Whether these two proteins are of the same nature remains to be solved by sequencing!

Mab 2 of Canada and mab 117/5 of France have been used in an inhibition ELISA assay.

Sera for inhibitions used were Bovine sera 448, 449, 450 as negative controls from the Omaheke region (CBPP free area) a positive control (PG_1, from the CBPP kit IAEA) and 4 positive sera. The wells were coated with 1 µg /well membrane protein (small colony)The sera were diluted 1/20 mab 2 and mab 117/5 at 1/40.

Only the positive control from IAEA inhibited. Good inhibition results were received of both mabs. Local bovine sera and sera from Botswana (weak and strong CFA titres) gave variable results.

APPENDIX 3

RENNES UNIVERSITY
FRANCE

SCIENTIFIC REPORT

Dr H. WROBLEWSKI

BRIEF REPORT FROM
UPRES-A CNRS 6026
UNIVERSITE DE RENNES 1 (FRANCE)
by H. WRÓBLEWSKI

INTRODUCTION

One of the most salient features of mollicutes is their unusually large content of membrane-bound, surface-exposed lipoproteins (For a review, see Wieslander et al., 1992). Among mollicutes, mycoplasmas can cause respiratory, arthritic and urogenital diseases in mammals including humans and several animal species of economical interest (notably cattle, pig, and poultry). During the early phase of infection, mycoplasmas usually induce inflammatory and humoral responses preferentially directed against their membrane lipoproteins. Furthermore, some of the variable surface antigens of mycoplasmas are lipoylated. Hence, a peculiar attention should be devoted to membrane lipoproteins in any work on the pathogenicity of mycoplasmas, on the interactions between mycoplasmas and the immune system of their hosts, and in the development of anti-mycoplasma vaccines as well as serodiagnosis techniques.

RESULTS

Membrane lipoproteins in *M. mycoides* ssp. *mycoides* SC (*Mmm* SC)

We have shown that the plasma membranes of *M. gallisepticum* (a poultry pathogen) and *Mmm* SC contain both 12 to 15 lipoproteins (Jan et al., 1995 and 1996). Similar to the membrane-anchored lipoproteins found in Gram-positive and Gram-negative eubacteria, the lipoproteins of these two mycoplasma species are di- or triacylated at the level of their N-terminal cysteine. In experiments made with chicken, we have shown that these surface antigens induce a strong and precocious synthesis of antibodies in the blood of infected animals (Jan et al., 1995).

In the specific case of *Mmm* SC, we have set up a procedure for the selective solubilization under nondenaturing conditions of membrane lipoproteins. This procedure is based upon the use of appropriate detergents. So far, we are able to copurify by HPLC, from the membrane lipoprotein fraction, two major lipoproteins (p42 + p65) of *Mmm* SC. However, in contrast to lipoproteins p52 and p67 of *M. gallisepticum* (Jan et al., 1996), the separation of p42 and p65 proves to be a difficult task, probably due to similar physical-chemical properties of these two antigens. Although it is possible to purify p65 at the 1 to 10-µg microgram level by micro-HPLC, this is still not enough for serious immunological investigations and structural-functional studies.

In spite of these difficulties, we have shown that the acylation of *Mmm* SC lipoproteins is selective, with palmitate (16:0) being the most abundant O-ester bound fatty acid and lionleic acid (18:2c) the most abundant N-linked fatty acid (Jan et al., 1996). We have also observed that the N-terminus of p65 resists Edman's degradation which is consistent with a triacylation of this lipoprotein.

Glycosylation in *Mmm* SC

Prokaryotes are capable of glycosylating lipids and some surface proteins such as S-layer proteins, pilin, and flagellin. All these proteins are absent in mollicutes but, owing to surface localization, membrane lipoproteins of these bacteria may be considered glycosylation candidates. However, there is so far no convincing report on the presence of glycoproteins in mollicutes. The only glycosylated compounds we could find in *Mmm* SC as well as in many other species, are glycolipids. Since *Mmm* SC contains a galactan capsule, any result suggesting the presence of glycoproteins in this mycoplasma should carefully verified using a very rigourous experimental approach.

Studies with spiralin

Spiralin, a well-characterized mycoplasmal lipoprotein, has been used as a model system to analyze the effects of a mycoplasmal lipiprotein on the mammalian immune system (Brenner et al., 1997). The great advantage offered by this protein is that we have developped procedures permitting to obtain large amounts (about 10 mg

per purification cycle) of perfectly pure, spiralin under a native conformation (Wroblewski et al., 1977, 1984, and 1989).

Purified piralin stimulated the *in vitro* proliferation of human peripheral blood mononuclear cells and murine splenocytes. The stimulation pathway was probably different from that followed by *Escherichia coli* lipopolysaccharide (LPS) because the effect of spiralin was not abolished by polymyxin B. Comparison of the effects of whole, native spiralin with those induced by proteinase K-digested spiralin or by the C-terminal half of spiralin (peptide p[13.5]$_T$) revealed that the first half of the protein, which contains the lipoylated N terminus, is responsible for the mitogenic activity. In contrast to whole spiralin, proteinase K-digested spiralin did not trigger murine B-cell differentiation and immunoglobulin G (IgG) and M (IgM) secretion. Stimulation of human and murine immune cells led to early secretion of proinflammatory cytokines (human tumor necrosis factor alpha and murine interleukin 1 or 6). Spiralin induced the T-cell-independent blastogenesis of murine B cells but did not stimulate T cells.

Altogether, our data demonstrate that spiralin possesses a potent immunostimulatory activity, similar to that reported for lipoproteins of pathogenic gracilicutes (gram-negative eubacteria; e.g., *Borrelia burgdorferi* OspA and *E. coli* Braun lipoprotein), are consistent with the fact that lipoproteins are major antigens during mycoplasma infections.

CONCLUSIONS AND PERSPECTIVES

The work performed by many research groups in the world clearly reveal the importance of membrane lipoproteins in the interactions between bacteria (including mollicutes) and their hosts. The data recently obtained by Mühlradt et al. (1996 and 1997) on the lipopeptide of *M. fermentans*, by Luke et al. (1997) on lipoprotein OspA of *Borrelia burgdorferi*, and Brenner et al. (1997) on spiralin show that membrane lipoproteins should be very seriously taken into account for the development of anti-mycoplasma vaccines.

Concerning CBPP, the goals of our research are to develop (i) a mg-scale purification procedure of lipoproteins p42 and p65 of *Mmm* SC and (ii) a large-scale

purification procedure of the lipioprotein fraction of this mycoplasma.

A the present stage of our research, it seems clear that the most difficult task will be the purification to homogeneity of p42 and p65 under nondenaturing conditions. In this purpose, we will try not noly to improve the HPLC-based method, but also use preparative isoelectrofocusing (PIEF). Concerning the large-scale purification of the lipoprotein fraction of *Mmm* SC, we have already set up a batch method which, owing to its simplicity, should prove easy to scaling up at the industrial level. It is noteworthy that we have managed to obtain ISCOMs from this specific preparation.

It will be interesting to compare p65 with the lipoprotein p72 described by Chen et al. (1996) as these two proteins might be homologous. In addition, we will study the topology of p42 and p65 in *Mmm* SC and determine the antibody accessibility of their epitopes on the cell surface. We will also determine the proteolytic susceptibility of p65 and other lipoproteins, as this is a crucial problem in oral vaccination.

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APPENDIX 4

CIRAD-EMVT, FRANCE

SCIENTIFIC REPORT

Dr F.THIAUCOURT

CBPP EU
Component 2
Workplan 2
F. THIAUCOURT

Most of the work performed during the workplan 2 was focussed on the use of the cELISA.

As co ordinator, I was very pleased to see that all the laboratories participating to this CBPP-EU project were able to perform some serological analysis as it is shown by the various reports. However it seems that the primary goals of the validation are not achieved yet.

One of these goals was to compare the real sensitivity and specificity of the CFT and cELISA in order to determine if the latter could replace the former one. It was absolutely necessary to gather reliable epidemiological informations on the sampled herds to do so and it seems that it was the most difficult thing to get. One of the point of interest was to determine the time elapsed between the contamination of the herd and the time of sampling as it is well known that the antibodies detected by CFT are short-lived, it would have been interesting to check if the cELISA detects antibodies during a longer time.

Another goal was to check if vaccination was inducing the production of antibodies detectable by cELISA. Up to now only Ethiopia achieved such a study and apparently antibodies are detected only for a short period, roughly one month. This result is very interesting as it indicates that cELISA could be used for the detection of outbreaks in countries that perform vaccination. Here again additional datas are needed to confirm these preliminary findings to take into account all factors that can infuence the antibody response. It is hoped that the various vaccine trials that should take place in the near future will help confirming these results.

Some very interesting results were obtained through the collaboration of the OVI in South Africa, thanks to Dr L Prozesky, although this Institute is not a member of the CBPP-EU project. Sera from infected cattle were analysed parallely at the OVI and the CIRAD. First of all there was a very good correlation between the results by the two Institute showing the robustness of the test. These serological results were afterwards compared with the results of the autopsy of the animals and the gross lesions that were observed. Upon 16 animals that had typical lesions of CBPP, 16 were found positive by CFT, 15 by cELISA and 11 by agglutination. Upon the 9 animals that had no visible lesions, one was positive in all three serological tests indicating that it had certainly been in contact with CBPP although no macroscopical lesions were detected. In conclusion of this study it has been shown that CFT and cELISA have similar sensitivities.

Additional results were obtained with sera from animals experimentally infected with a European strain of MmmSC, thanks to Pr Nicolet from the Bern University. Although few animals were available in this study, kinetics of antibody rise were performed. They showed that the cELISA titers remained highly positive in the only animal that had developped lesions whereas the other two, in which no lesions were detected at slaughter, had an antibody kinetic that was only transient both in CFT and cELISA. The animal that was not contaminated had negative titers throughout the experiment.

A training course has been held under the aegis of the AIEA at Onderstepoort Veterinary Institute. You will find the report attached. This training course enabled a great number of technicians from the southern part of Africa to become familiar with the cELISA.

It permitted us to finalyse the protocol that will be distributed with the kit. Especially important is the mixing of the diluted sera and the Mab before the transfer in the coated plates. This ensures that the test is really a competition and it should permit to obtain more consistent results with the control negative.

As a conclusion I would like to sinsist once again on the importance of obtaining accurate epidemiological datas on the animals that are investigated by CFT and cELISA. Therefore all efforts should be made to return regularly in herds where CBPP has been detected in order to perform sequential analysis at many months of interval on the same animals.

FAO
AIEA- Vienna (Austria)

Competitive ELISA for CBPP

Training course at Onderstepoort Veterinary Institute

Republic of South Africa, June 23rd- June 27th 1997

F. THIAUCOURT
CIRAD-EMVT

August 1997

I) INTRODUCTION

Contagious bovine pleuropneumonia has been known for decades in Africa, it was under control until 1990 but since then it has spread to countries which were previously free. In the southern part of Africa it can be considered as an emerging disease and a threat to cattle raising for many countries. Two enzootic foci exist in the West (Northern Namibia and Angola) and in the North East (Tanzania). From there, CBPP might well expand to neighbouring countries. It seems to be the case in Zambia and it has already happened in Botswana. This later country regained its "freedom status" by a drastic slaughtering policy of about 320000 cattle.

Detection of outbreaks is an essential step to control the disease. Serological methods include the CFT which is now the official test for international trade. This test was used extensively in Australia during the eradication program and it proved very useful. Its main advantage is to permit the detection of acute CBPP outbreaks in countries that vaccinate with live vaccines, as this type of vaccination elicit variable but low amounts of antibodies which disapear after three months. It has however a number of drawbacks. It needs trained technicians, it is sometime difficult to standardize due to the use of fresh sheep red blood cells, it permits the detection only of recent outbreaks at the herd level, false positive results might exist. Therefore it can be considered as a good confirmation test enabling to evaluate the incidence of the disease but not the true prevalence in a country.

A new serological test, a competitive ELISA, has been designed recently (LeGoff and Thiaucourt, submited for publication). It was designed primarily to improve the specificity through the use of a specific monoclonal antibody, it later proved to be able to detect antibodies much longer than the CFT. It was therefore decided to introduce it in the field through the AIEA laboratory network. A first training course had been held in Montpellier for countries involved in the CBPP-EU research project, this second workshop was devoted mainly to southern African countries.

II) MATERIALS AND METHODS

All equipments were provided by the OVI or purchased by the IAEA for this training course (Multichanel pipettes and tips). The cELISA CBPP kit was provided by the CIRAD-EMVT, it was the same batch that was used throughout the validation of the test. Tested sera included one hyperimmune serum from CIRAD and four freeze-dried sera from the OVI . Three of them were positive sera obtained through the courtesy of Botswanian Veterinary Services (A,B and C) and one negative serum from RSA (D). A modification of the procedure was included during this workshop: sera were diluted by adding 11µl to 100 µl of diluent and the monoclonal antibody was added in the same plate (110µl) before the mixture was transfered to the coated plates (100µl per well). Plate readings were made by Dr R. GEIGER with the EDI software distributed by the AIEA. All plates were coated ovenight at 4°C.

The nineteen participants came from: Benin, Botswana, Ethiopia, Ghana, Kenya, Mali, Mauritania, Namibia, Niger, Nigeria, South Africa, Uganda, Tanzania, D.R. of Congo, Zambia.

III) DAY ONE: Use of multichanel pipette

For the first test, all reagents were prepared beforehand by the same person in order to minimize the variability of results between groups.

One hyperimmune serum (Afade), diluted 1/10, was provided to all groups. This serum was diluted by the participants in 9 succesive twofold dilutions by a multichanel pipette (8 rows) set at 50µl. All dilutions were tested in cELISA. Parallely the four sera from the OVI were tested.

Afade 1/10

Cc	Cc		20	40	80	160	320	640	1280	2560	A
C++	C++										A
C++	C++										B
C+	C+										B
C+	C+										C
Cm	Cm										C
Cm	Cm										D
C-	C-										D

Table 1: plate layout of the first day

RESULTS

Dilutions

The results are presented in nine graphs showing the optical densities obtained with each of the dilutions.

Sera

	A	B	C	D
Mean	35,8	64,5	59,9	10,6
Stds	6,9	4,6	4,8	9,4
n	8	8	8	8

Table 2: Results of the 4 sera tested the first day

DISCUSSION

Dilutions of serum

All groups obtain similar results ie an optical density around 0,3 for the less diluted serum

and an optical density of around 1 for the most diluted serum. These values are normal as this is a competition assay and positive sera inhibit the fixation of the monoclonal antibody.

However it is obvious that the acuracy of the pipetting varied greatly between the groups. The best results are obtained by group N°1, almost no difference is observed between the eight duplicates whatever the dilution. Various errors can be observed in the other groups.
Absence of monoclonal antibody leads to a very low individual result (Group 8, 11th dilution). A single error of pipetting at the beginning give rise to parallel curves (Group 8). Loose attachment of some tips result in some curves being erratic when all others follow the normal pattern (Group 7). Addition of errors lead to very dispersed results (group 4).

This first practical exercise shows clearly the importance of technician training. During this first day the single cause of variation was the technician's skill as all reagents had been prepared beforehand and all pipettes and tips were brand new. The results of group N°1 shows that trained technicians can obtain almost perfec results, however, in the field, other causes of variations may exist such as poor maintenance of the multichanel or re-use of tips.

Field sera

The negative serum from South Africa has a very low value (10,6%) which is satisfactory, as far below the cut-off point set at 50 percent. Among the three sera that were positive by CFT only two are positive by cELISA (B and C). This is not surprising as high positive CFT titers may be due to early serological response with IgM that have a low affinity and may recognize other epitopes than the one recognized by the monoclonal antibody used in this test.

The standard deviations of the final results between the different groups are quite low in spite of the fact that some technicians had no previous training with this test or any competition assay.

IV) DAY TWO: Repeatability of the test

For this second test, various sera were tested by all participants (n=19). Here again the different solutions were prepared beforehand. The sera were given pure, already dispached in a microplate, so that each participant had to perform the dilutions, adding one cause of variation compared to day one.

For this trial, various "artificial sera" were obtained by making dilutions of positive sera (Afade, B and C) in order to obtain the whole range of positivities. The highly positive control was replaced by the slightly positive one in order to increase the number of duplicates. Completely abnormal values, usually due to omission of Mab, were not taken into acount in the calculations.

RESULTS

Distribution of results for the slight positive control

The distribution of results was based on 17 individual technicians, two having completely abnormal results were not included. Each technician tested the same serum eight times, hence a total of 136 results. The distribution is gaussian as seen on fig. 2. It is therefore permitted to calculate the mean and standard deviation of the sample.

Mean = 47,4 Stds =7,1 n =136

Distribution of results for individual sera: inter-technician variations

Each of the serum having been tested by 19 technicians, it is possible to evaluate the inter-technician variations. This can be assessed for each serum by calculating the standard deviation of the 19 duplicates. The STD depends on the value of the serum titer, as shown on an xy graph (Fig 3). The correlation is linear but values are widespread around the line.

Another xy graph was plotted by excluding the plates with a high number of sera with differences between duplicates superior to 10 or that had values constantly and significantly superior to the expected results. Four plates were excluded and it improved greatly the distribution of results (Fig 4). There is a linear correlation between the standard deviation of a result and the percentage of inhibition. Sera with PI values around 35 have a std reaching 5, sera with PI values above 55 have a std value of 3.

DISCUSSION

In competitive ELISA for CBPP, there are obvious causes of errors. The most frequent one is a mishandling of pipette or tip that lead to an inacurate volume of serum or monoclonal antibody. As this error occurs randomly, it will result in a high difference between the two duplicates of a serum and it will be spotted easily. From previous experience, it was known that the standard deviation of a result should not exceed 2,5 (same laboratory, same technician, same day), therefore the difference between two duplicates should not be greater than ten.

Counting the number of sera, within a plate, that fall outside this limit permits to evaluate the performance of the technician and the confidence that can be given to the results. It is advisable to exclude the individuals results if duplicates differ from more than 10 and to exclude the plates where more than 4 sera are outside these limits. This procedure improved the correlation between the STD of a value and the PI of the sera (Fig 3 and 4). Four plates out of 19 were excluded.

Other causes of variation are more difficult to spot and will slighly decrease the accuracy of the measurement. This training course enabled us to calculate the standard deviation of results due to inter-technichian variations. As expected, it varies according to the PI of the serum, reaching 5 for a serum having a PI of 35 and 3 for a serum having a PI of more than 55. One possible explanation for these variations of STD values could be linked to the quantities of epitopes available. When there is an excess of bovine antibodies (high PI), slight volume differences will not alter the result dramatically and the final PI will depend mainly on the affinity of these antibodies and their ability to compete with the Mab for the fixation to the epitope.

The criteria for serum result acceptance should be based only on the values of the duplicates. In any case, it is quite normal to have duplicate values falling on either side of the cut-off if the real PI is equal to 50.

V) DAY THREE

For this final testing, the participants prepared all the reagents and performed all the dilutions. Each technician was given 20 sera to test (one plate per group).

RESULTS

Reference sera

All plates had acceptable values for the different controls. The required OD in the Cm was obtained in twenty minutes approximately, the lowest value was 0,97 and the maximum 1,5. The values of the Cc never exceeded 0,1 (one plate) and had an average value of 0,035. The control sera had acceptable values (Tab 3). Here again there is a correlation between the percentage of inhibition and the Std of the mean (fig 5)

	C++	C+	C-
Mean	75,4	48,9	11,2
Stds	2,2	2,9	5
n	32	32	16

Table 3: Values of the control sera during day 3

Only one plate had to be rejected as it had two many duplicates falling outside limis (difference higher than 10). On closer look, it was obvious that the error was due to a mishandling of the multichanel pipette as the error was repeated on all sera tested in raw A and B.

Value of the difference between duplicates

This value was calculated on 317 sera, the mean is -0,54 and the Std 2,8 (three values were removed as completely outside limit). When observing the distribution of the absolute value of the difference, which is given by the EDI program, it is clear that it is a unilateral distribution and that the three outside limit values cannot be explained by random variation (fig 6)

Inter-technician variations

Two sera, one positive and one negative were analysed by the different technicians (10 groups, 2 technician per group, 5 replicates per technician). The distribution of the result is gaussian as shown on fig . The final resultsare shown in tab 4

Serum	A	B
Mean	67,2	27
Std	1,7	3,7
n	100 (5x20)	100

Table 4: Values of two sera when tested by the 20 technicians

DISCUSSION

This last testing gives the real figures of what can be expected from this cELISA test in the field first because the technicians were trained for two days previously and therefore were accustomed to the test and second because the test was run by each technician without any exterior help.
Results were very satisfactory.

All plates fell within the acceptance limits and the twenty sera tested gave similar results whatever the technician (9 negative, 11 positive).

The value of the difference between duplicates were very low, usually less than five, showing that all technicians performed the test correctly. The few duplicate values that fell outside the 10% range were easily spoted and due to problems in the transfer of monoclonal antibody.

The inter-technician variations gives an indication of the robustness of the test. The standard deviation of a result, when calculated by pooling results from all the technicians, progressively decreased day by day. This shows that the ability of the trainees was significantly increased during this workshop. The final values obtained at day 3 are only slightly higher than the values that are normally obtained at CIRAD. For a highly positive serum (67%) the Std reaches 1,7 and for a negative one (27%) it reaches 3,7.

VI) CONCLUSION

This training course was the first step to the introduction of this new serological method for CBPP in the southern part of Africa. All trainees became easily familiar with the test and should be able to implement it in their respective countries provided that the standard facilities for ELISA are available.

Some slight modifications in the protocol for serum dilutions have been tested during this workshop and proved satisfactory. The mixing of the monoclonal antibody with the diluted serum before the transfer to the coated plate give less variable results than when adding the monoclonal antibody afterwards. With the new protocol all participants had normal values with the controls and especially with the negative one that should have a percentage of inhibition below 25.

In the future it seems advisable that the EDI program should take into account the values of duplicate difference as a criteria for single serum acceptance (difference less than 10) and also plate acceptance (less than 5 serum rejected). This measurement is a very good indication of the performance of the test.

Finally, I wish to thank the team of the O.V.I. for its kindness and helpfulness and also Dr R. Geiger, also for his kindness, but also because he performed all the ELISA readings and transfer of data.

APPENDIX 5

LANAVET - GAROUA CAMEROON

SCIENTIFIC REPORT

Dr A. YAYA

LANAVET

LABORATOIRE NATIONAL VETERINAIRE

BP 503 GAROUA, CAMEROUN. Tel.. (237) 27 13 05 / Fax: (237) 27 15 42

EU- CBPP RESARCH PROJECT

REPORT OF THE SECOND SEMESTER

WORKPLAN II, Component 2

YAYA Aboubakar
HAMET Moussa

Introduction

The National Veterinary Laboratory (LANAVET) is involved in a research program on contagious bovine pleuropneumonia. This program, supported by the European Union, is mainly composed of 3 elements:

- component 1: development of ISCOM vaccine ;
- component 2: improvement of diagnosis tools ;
- component 3: immunology of the disease.

Work on components 2 and 3 has been carrying out since the program has been launched. Experimentations on ISCOM Vaccine are expected to start very soon.

This report will present results obtained on work carried out in LANAVET on component 2 during the past 6 months (second semester, Workplan II).
The current situation of CBPP in Cameroon will also be given in this report.

Materials and methods

1° - Experimental animals

Animals of the stable were composed of 3 groups:

- group I: animals of this group were infected on May 12th and 13th, 1997 by intra-tracheal route with *Mycoplasma mycoides subsp mycoides* SC, Afadé strain. Their tag ear numbers were: I_0 , I_1 , I_2 , I_3 ,I_4, I_5, I_6, 855 and 857. The titration on the day of infection gave a titre of 10^7 Mycoplasma per ml. Each animal received 50 ml of culture mixed with 30 ml of agar (1.5 %). 100 ml of culture medium (Heart Infusion Broth) were after added.
Animals numbers 855 and 857 had been used last year as contact animals during the first experimentations conducted in LANAVET and at the end of the period of observation, they were still negative in serology.
One month later, as only few infected animals displayed symptoms, they were once more infected in the same way with a culture which titer was 3.10^8 CCU per millilitre.

- group II: a group of 15 animals was put in contact with the first group three days after infection. Their tag ear numbers were: C_1, C_2, C_3, C_4, C_5, C_6, C7, C_8, C_9, C_{10}, C_{11}, C_{12}, C_{13}, C_{14}, C_{15} and C_{16} (C_1 has died and was later replaced by another bull called C_{16})

- group III: Two animals were used as controls: T_3 and T_4.

2° - Samples

The following samples were received at regular intervals (once per week for infected animals and every two weeks for others):

- broncho-alveolar lavages (BAL) ;
- nasal swabs ;
- sera.

3° - Tests performed.

The following tests were performed:

- bacteriology: titration of Mycoplasma in BAL ;

- recovery of Mycoplasma from nasal swabs ;
- isolation of Mycoplasma from lungs and pleural fluids after death.
- antibodies titration by Complement fixation test (CFT) and competitive ELISA.

The ELISA kit was provided by CIRAD-EMVT.
Sera were tested in CFT for the dilutions of 1/10, 1/20 and 1/40. As recommended by the workshop held on October 1996 at Montpellier (France), sera were tested without inactivation.
Mycoplasma were titrated on microplates and on Petri dishes. HIB (Heart Infusion Broth with red phenol) and HIA (Heart Infusion Agar, Difco) were used as culture media.
Antigen, monoclonal antibody, conjugate, control and test sera were diluted as recommended by the manufacturer (CIRAD-EMVT). Plate Polysorb #269620 were used (NUNC, Denmark).
In C-ELISA, 45 % of competition was retained as the positive threshold. In CFT, sera showing complete fixation of complement at 1/10 dilution were recorded as positive.
BALs were diluted in 10 fold from 10^{-1} to 10^{-10}. 100 µl of each dilution were distributed in 10 wells of microplate containing 100 µl of culture medium (HIB) with red phenol. 20 µl of each dilution were also deposited on Heart Infusion Agar.
After one week to 10 days of incubation, the number of wells which colour changed from pink to yellow and the number of colonies in HIA were recorded. Contaminated microplates were discarded by observation of fresh preparations on light microscope.
After sampling, nasal swabs were soaked in 2.5 ml of HIB medium and Mycoplasma were titrated. The objective was to compare the quantity of Mycoplasma that animals eliminated from their nostrils and their kinetic.

Results

Bovines I1 and C11 died very soon and the death was not related to CBPP. Results of these animals are not given in this report.

Group III: in group III, two animals used as negative controls have been found negative in serology and no Mycoplasma was recovered from BALs and nasal swabs.

Serology

- Group I

In group I, all animals became positive in competitive ELISA and CFT. All those animals were positive from 9 days to 2 weeks after infection.

The following figures present the evolution of CBPP antibodies with competitive ELISA test in infected group.

Figure 1: kinetic of CBPP antibodies in C-ELISA. Animal number I_0

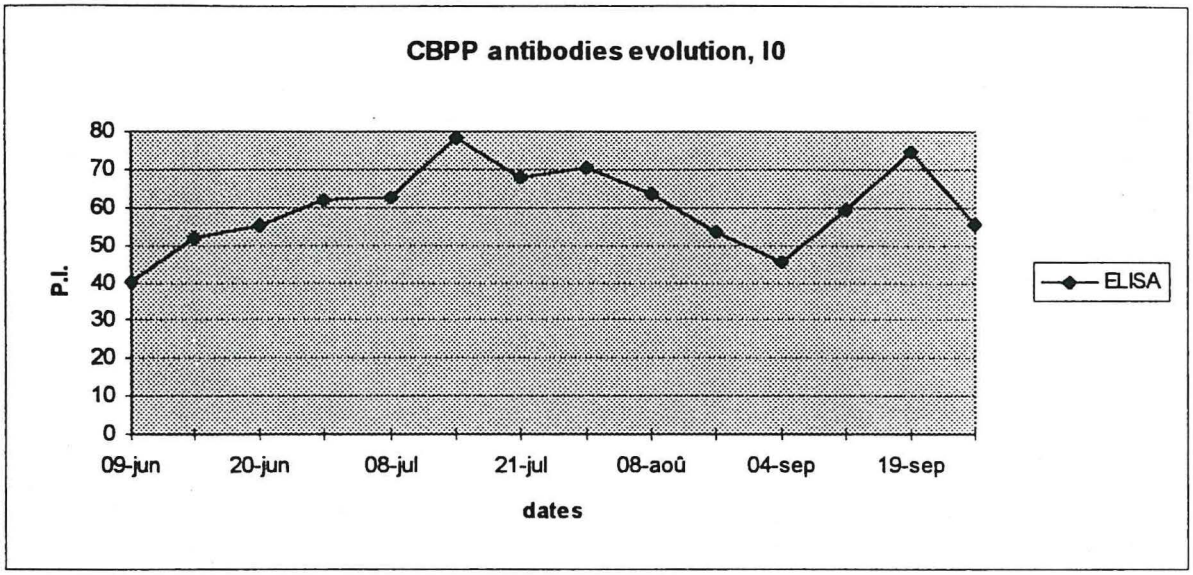


Figure 2: kinetic of antibodies in competitive ELISA, animal I_2.

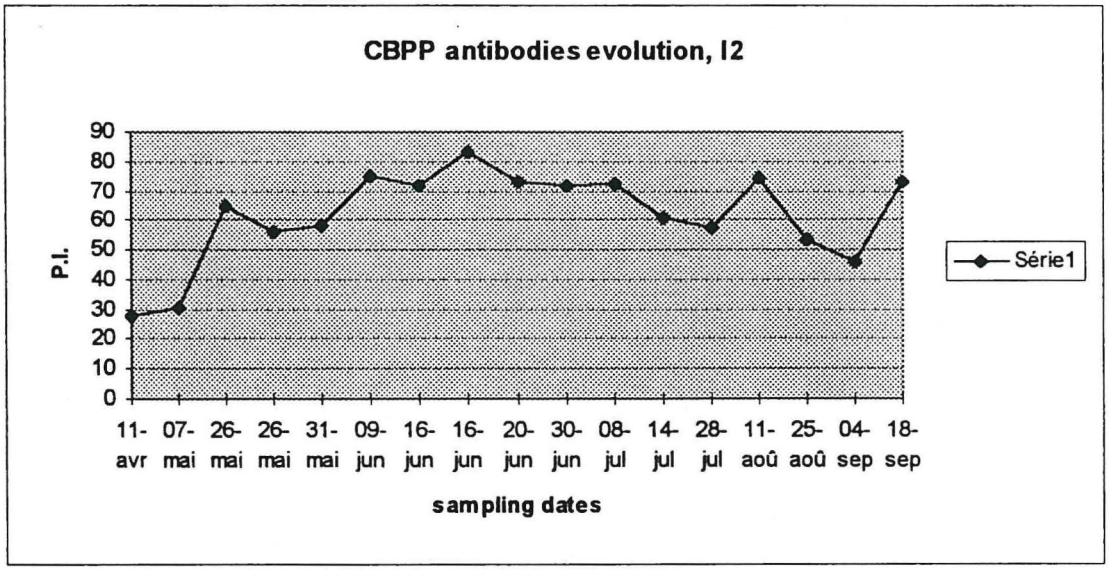


Figure 3: kinetic of antibodies in competitive ELISA, animal I_3.

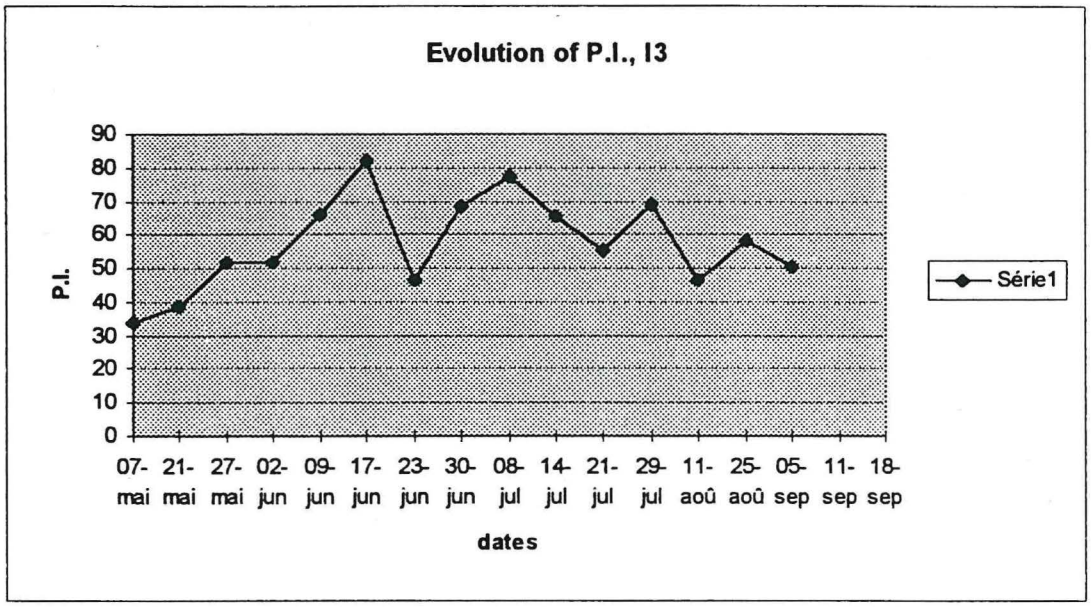


Figure 4: Kinetic of antibodies in competitive ELISA, animal I_4.

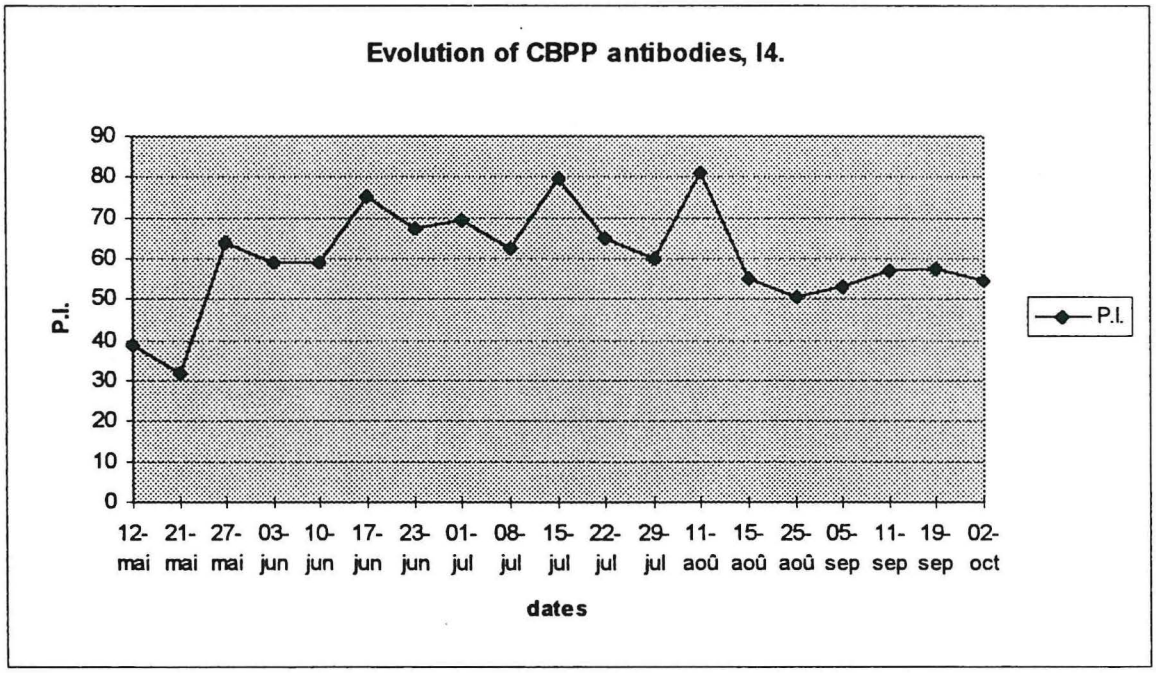


Figure 5: kinetic of CBPP antibodies in C-ELISA, infected animal number I5

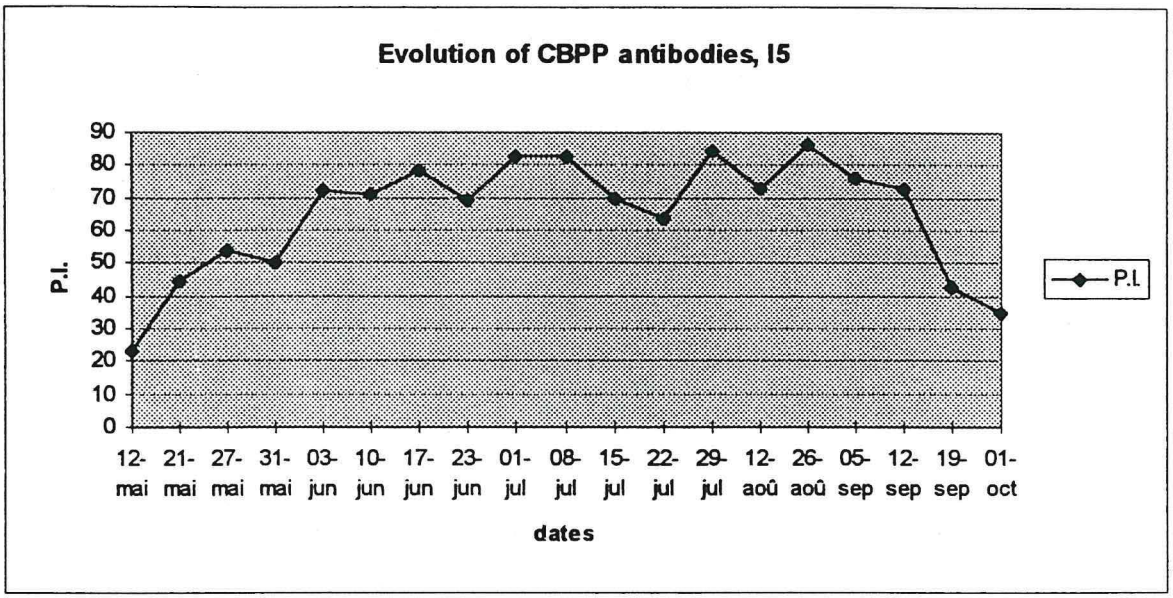


Figure 6: Kinetic of CBPP antibodies in C-ELISA, Infected animal number I6.

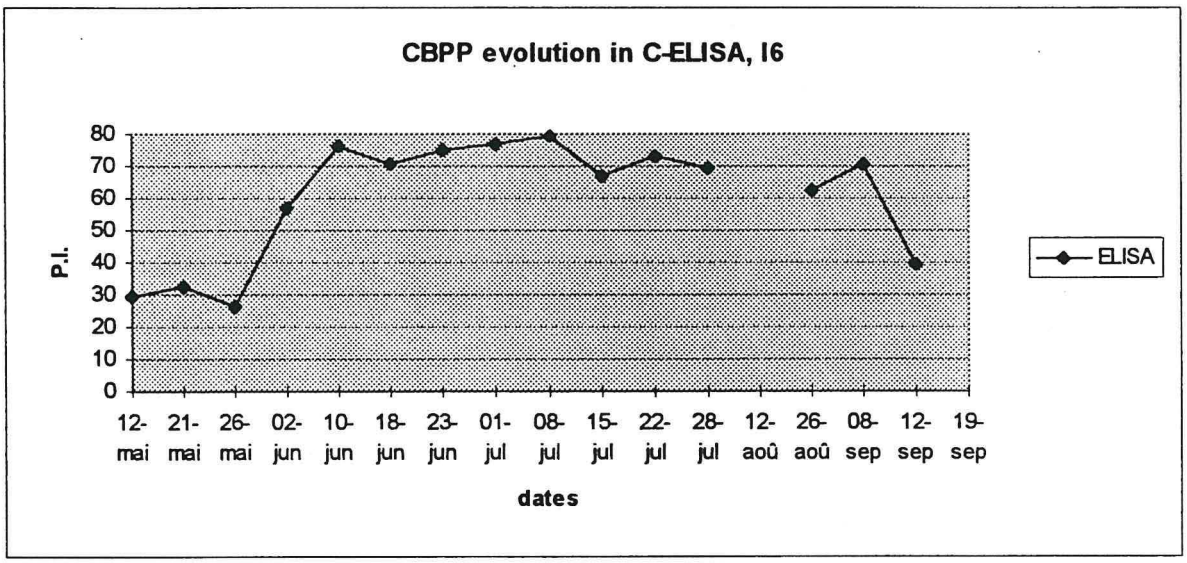
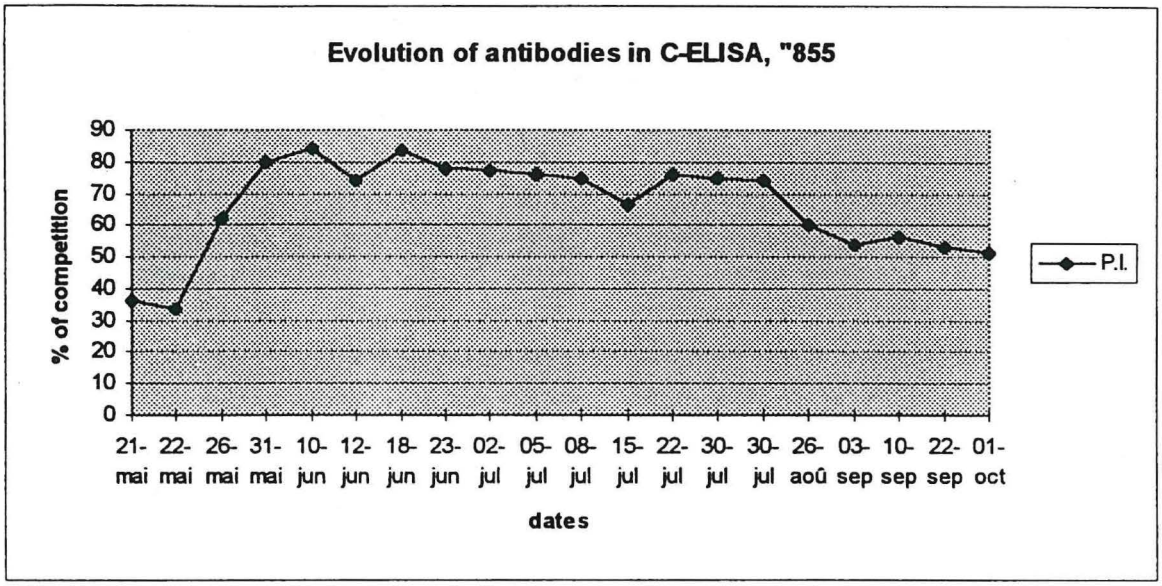


Figure 7: Kinetic of antibodies in competitive ELISA, infected animal n° 855.



This bull was used in 1996 as contact animal and was negative in both CFT and competitive ELISA tests.

- Contact group

Among 15 cattle put in contact with group I (C_1 died a few days later and has been replaced by C_{16}), 12 became positive in both CFT and competitive ELISA. Seroconversion occurred from week 5 to week 14 after the contact was made. Their ear tag numbers were: C_2, C_3, C_4, C_5, C_6, C_7, C_8 , C_9, C_{10}, C_{13}, C_{14}, C_{15}, and C_{16}. Results of C-ELISA and CFT were similar except C_{14} was negative with the latter test.

Animal C6 became positive 7 weeks after contact (in C-ELISA and CFT) but the animal very soon became negative. The percentage of inhibition remained under the threshold of positivity (45 %).

At the end of the period of observation (3/10/1997), all contact animals were positive in serology except number C6. The latter has shown seroconversion only for a period of 5 weeks.

Three remaining cattle of this group (C_1, C_{11}, and C_{12}) were negative during the period of observation (ELISA and CFT).

Kinetics of CBPP antibodies for some contact animals are given below.

Figure 8: kinetic of CBPP antibodies in C-ELISA, contact animal number C_{10}.

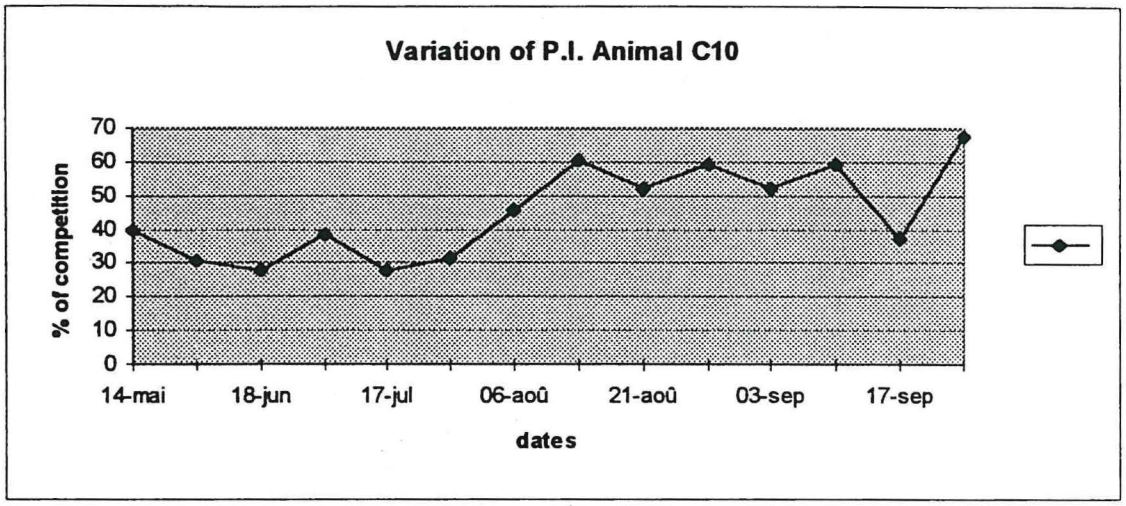


Figure 9: kinetic of CBPP antibodies in C-ELISA, contact animal number C_5.

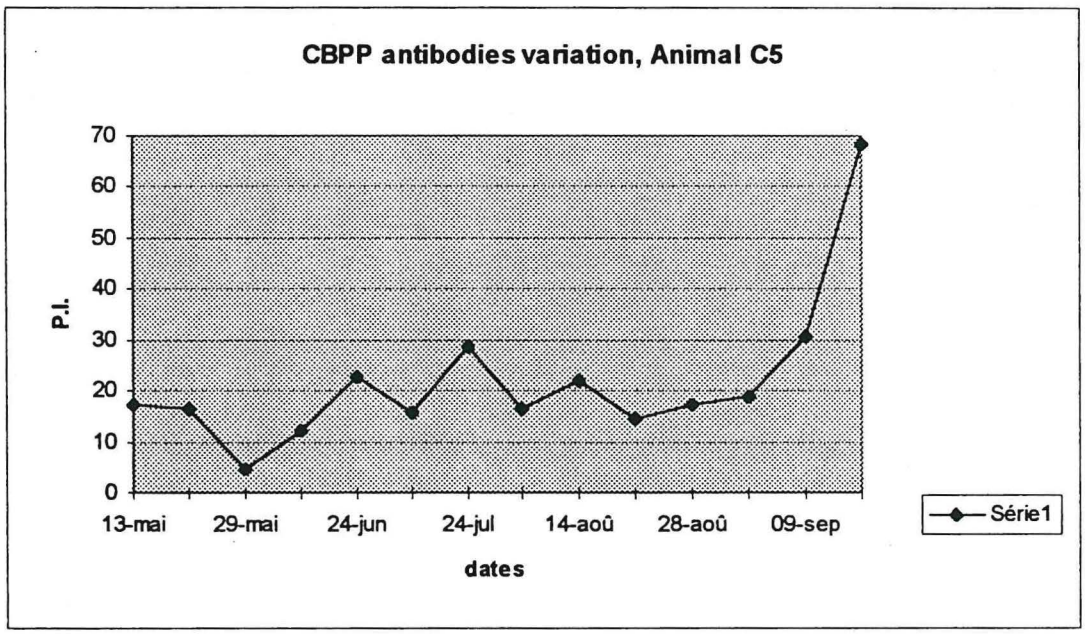
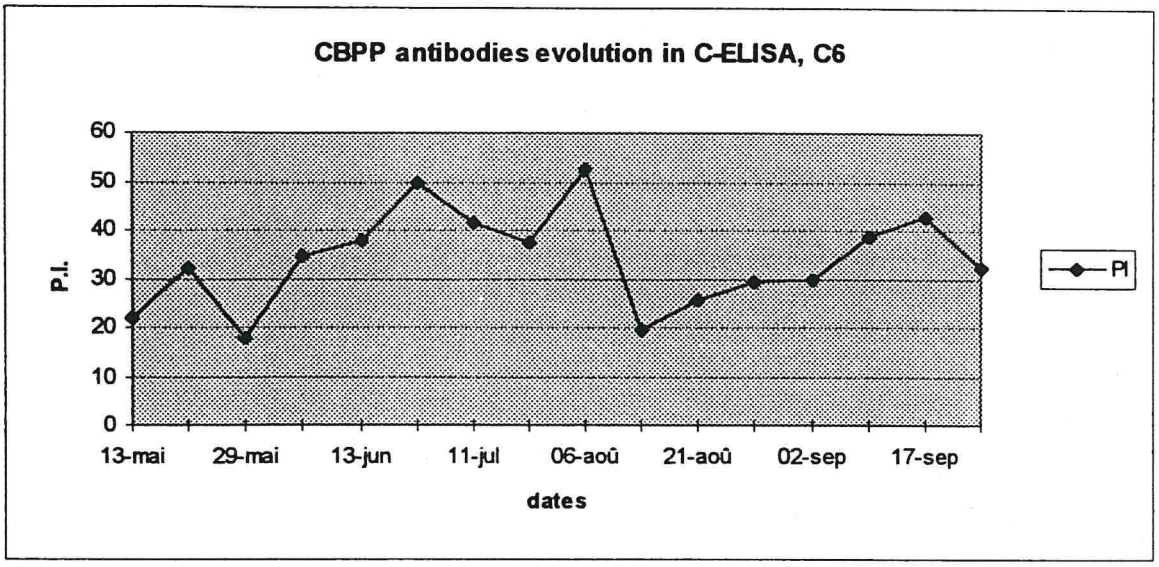


Figure 10: CBPP antibodies in C-ELISA, contact animal number C_6.



Control values in Competitive ELISA

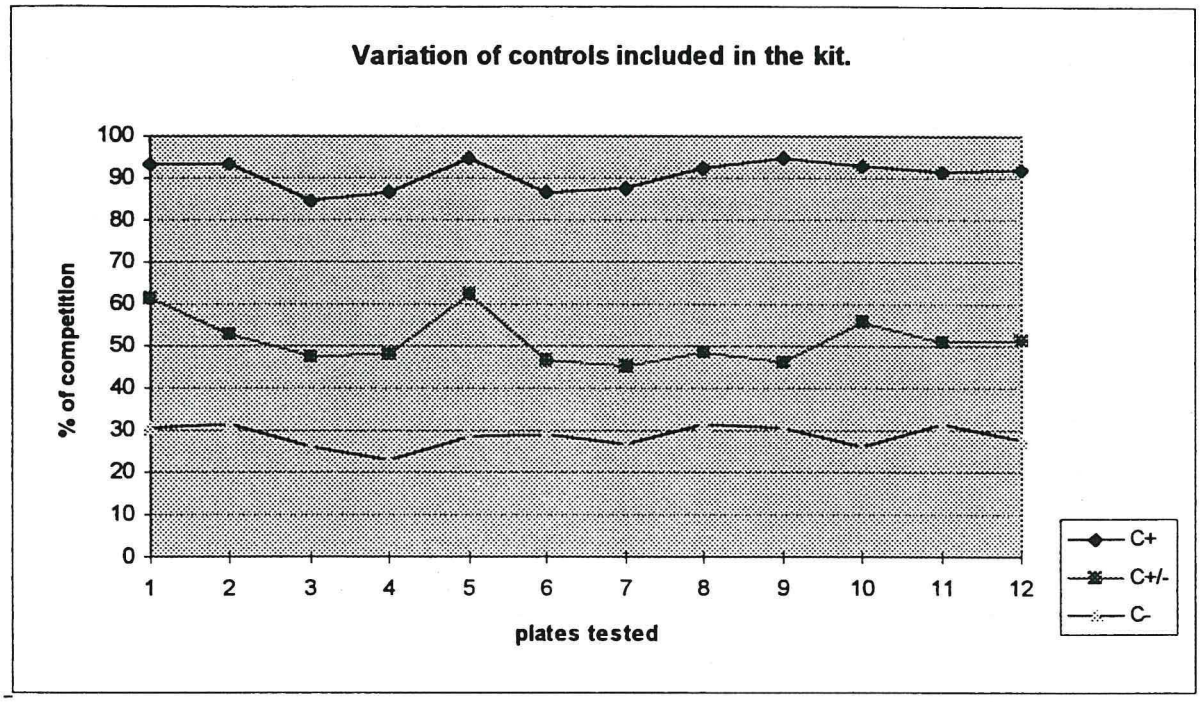
Values on strong positive, weak positive and negative control sera are given in the following table for twelve plates :

Table 1: parameters of control sera included in C-ELISA kit.

Control sera	Mean % of competition	standard deviation	Variance	Maximum value	minimum value
C++	90.8	3.45	11.91	94.7	87.7
C+/-	51.4	5.83	34.00	62.6	45.3
C-	28.6	2.69	7.28	31.5	22.9

Those variations are represented in the above figure.
The following figure presents the variation of three control sera (strong positive, weak positive and negative sera). Results of rejected plates are also included. Twelve plates have been analysed. More variations were obtained with weak positive control sera.

Figure 11 : variation obtained with control sera included in C-ELISA kit.



Bacteriology:

1° - titration of Mycoplasma in broncho-alveolar lavages (BAL).

Mycoplasma mycoides mycoides SC were recovered from BALs of seven infected animals. (855, 857, I0, I1, I2, I3, I6). Mycoplasma were recovered during the first 10 weeks post infection. No Mycoplasma was further isolated after this period.
No positive results were obtained with samples of contact animals (group II).
The following table gives results of Mycoplasma titration in BALs.

Table 2: titer of Mycoplasma in BALs and dates samples were collected.

Ear tag number	**dates of sampling**	**titer per millilitre (log_{10})**
855	June 2 nd	3.65
857	May 21	6.75
857	May 27	6.45
857	May 27	6.45
857	June 4	7.35
I_0	June 20	6.7
I_0	July 22	7.39
I_1	May 26	3.95
I_2	May 26	3.95
I_2	July 22	6.87
I_3	May 21	2.45
I_3	July 22	6.87
I_6	May 21	3.45

2°- research of mycoplasma from nasal swabs

From nasal swabs, *Mycoplasma mycoides mycoides* SC were recovered only from two animals (857 and I1).

Results on isolations performed on samples got after post mortem examinations are summarised in the table below.

Table 3: summary of results (serology and bacteriology)

Ear tag number	**CFT**	**C-ELISA**	**Bacteriology after post mortem exam**	**Dates of death or slaughter**	**Comments**
855	+ after 2 w.	+ after 2 w.	contaminations	1/10/97	
857	+ after 2 w.	+ after 2 w.	No sample		
I_0	+ after 2 w.	+ after 2 w.		30/09/97	
I_1	+ after 2 w.	+ after 2 w.	+	31/5/97	
I_2	+ after 9 days	+ after 9 days	+	1/10/97	
I_3	+ after 2 w.	+ after 2 w.	Not performed	still alive	
I_4	+ after 2 w.	+ after w.	+	2/10/97	
I_5	+ after 2 w.	+ after 2 w.	contaminations	1/10/97	
I_6	+ after 9 days	+ after 9 days	not performed	still live	
C_1	-	-	not performed	?	death not related to CBPP
C_2	+ after 14 w.	+ after 14 w.	+	2/10/97	
C_3	+ after 14 w.	+ after 18 w.	contaminations	2/10/97	
C_4	+ after 13 w.	+ after 15 w.	Not performed	still alive	
C_5	+ after 13 w.	+ after 17 w.	Not performed	still alive	
C_6	+	+	contaminations	3/10/97	
C_7	+	+	+	14/08/97	
C_8	+ after 8 w	+ after 8 w.	+	14/07/97	
C_9	+ after 5 w	+ after 7 w.	+	14/07/97	
C_{10}	+ after 11 w.	+ after 9 w.	-	3/10/97	
C_{11}	-	-	not performed	16/05/97	death not related to CBPP
C_{12}	-	-	+	3/08/97	
C_{13}	+ after 12 w.	+ after 12 w.	+	8/08/97	
C_{14}	+ 9 w.	-	samples not received	?	
C_{15}	+ after 11 w.	+ after 7 w.	+	25/08/97	
C_{16}	+ after 9 w.	+ after 9 w.	-	29/09/97	
T_3	-	-	not performed	still alive	
T_4	-	-	not performed	still alive	

*: w = weeks

Situation of CBPP in Cameroon.

In this year (1997), outbreaks have been reported in two provinces of Cameroon: North and Far-North provinces. Those provinces are pastoral regions near the Nigeria and Chad borders.

- Demsa outbreak occurred in February 1997. A field trip has been organised and samples collected. *Mycoplasma mycoides subsp. mycoides* SC has been isolated.

- At Mora in far-north province, veterinarians have described a typical case of CBPP with acute lesions. As the laboratory didn't receive appropriate samples, no Mycoplasma was isolated. But some sera from one affected herd have been analysed and were found positive in Complement fixation test. As this herd was not recently vaccinated, the diagnosis was confirmed.

- The third outbreak has been reported in Garoua neighbourhood. Two animals died with acute lesions of CBPP. Investigations were carried out in serology and bacteriology. 36 % of animals were positive in C-ELISA and/or in CFT. *Mycoplasma mycoides subsp. mycoides* SC has been isolated.

- Another case of CBPP has been reported at Goulfey in Logone and Chari division (in lake Chad area). The veterinary officer has described acute lesions of CBPP, but no samples were refereed to the laboratory.

Discussions

All infected cattle developed specific antibodies within two weeks post-infection. Animals infected with 10^7 Mycoplasma did not display symptoms very soon but they all developed antibodies as evidenced by C-ELISA and CFT. The delays within antibodies appeared were the same than those found during the trial in 1996.

The expected results were not obtained in bacteriology. It was not possible to have the kinetics of elimination of Mycoplasma during the period of observation. Only few animals shared Mycoplasma. This situation is not true as it was seen by serology and clinic. Three reasons can be given:

- the replacement of the trained technician by another, specially in medium preparation ;
- the use of a batch of imported horse sera which quality was not satisfactory ;
- and the presence of aminosides in broncho-alveolar lavages (antibiotics which are mycoplasmicid) .

Only few sporadic cases restricted to the northern regions have been reported. There is no systematic surveillance of CBPP for the moment in Cameroon. Then, it is not possible to have real prevalence, and to know the extension of the disease or its control.
During the years 1970, by combining slaughter policy, vaccination and movement control, the disease was eliminated from some regions in Cameroon (Adamaoua province). This region is still free of the disease. In other areas, the situation is relatively controlled by vaccination. It is important to implement an adequate system of surveillance of CBPP in Cameroon.
An adequate surveillance of CBPP must include:

- serological surveillance and clinical diagnosis ;
- post-mortem examination at abattoirs ;
- investigation of herds where there is suspicion ;
- expedition of samples to the laboratory in case of any suspicion.

Results on Mycoplasma isolation from sequester are still awaited.

Perspectives for Workplan III

For the third year of the project in LANAVET, animals experimentations will not be conducted like during workplan II. But experimentations on ISCOM vaccine are expected to start very soon. Bovines shall be vaccinated with ISCOM vaccine and put in contact with infected animals. Those animals in contact shall be followed up in serology (CFT and C-ELISA) and bacteriology. The duration of humoral immune response will be follow up.

As LANAVET is involved in another project related to CBPP (to compare the efficacy of two vaccinal strains, T1-SR and T1-44), a herd of infected cattle will be present and shall be used for the contact with vaccinated group.

Conclusions

The LANAVET participated in the research project on CBPP for the second year. A group of 6 cattle were infected and put in contact with a healthy group. Those animals have been followed up in serology and bacteriology. All infected animals and 12 contact animals were positive in serology. *Mycoplasma mycoides mycoides* SC was isolated from BALs, nasals swabs, lung and pleural fluids. But it was not possible to have the kinetic of mycoplasma's excretion from the respiratory tract.

APPENDIX 6

N.V.I. DEBRE ZEIT
ETHIOPIA

SCIENTIFIC REPORT

Dr L.M. YIGEZU
Dr F. ROGER

CBPP/EU project
Annual report workplan N0 2, Nov, 1997
NVI, Debre Zeit,

Introduction'

This year's CBPP project activities include testing of additional bovine serum samples from North-west Ethiopia and evaluation of sero-conversion and duration for T_1-SR and T_1 44 vacciñes, in ranches and dairy farm.

The comparison of the Compliment Fixation and C-ELISA tests continued with participation of one post-graduate student Gashaw Takele in Tropical Veterinary Epidemiology / joint programme Freie Universitat of Berlin and FVM Debre Zeit /. A total of 3150 bovine sera, out of which 2260 were randomly collected from 39 selected villages in 11 districts of Awi and west Gojjam zones /North-west Ethiopia/. The rest of the samples are from vaccinated cattle. The surveyed districts are Ankesha, Banja, Dangila and Guangua from Awi and Achefer, Bahir-Dar, Burie, Denbecha, Jabi-Tenan, Mecha, and Sekela from west Gojjam. Some of these districts included in the epidemiological survey were known as endemic area for CBPP.

In an attempt to compare the efficiency of the C-ELISA with the CFT in relation with evaluation of the post-vaccination antibody response and its duration, 120 sera from some vaccinated population under extensive management, from two government owned cattle breeding ranches (Metekel & Andassa) and 770 sera from dairy farm at Debre Zeit Research Institute were tested.

Vaccination of cattle:

Vaccinated cattle from two districts namely; Jabi-Tenan and Denbecha were sampled at about four months of vaccination. The vaccine was applied by the respective district animal health professionals as a prophylactic measure against CBPP during occurrence of ourbreaks in the adjacent districts. The vaccine strain used was T_1- SR freeze dried attenuated mono-valent vaccine $10^{7.7}$ CCU_{50} per dose, batch number PL17-94 and the route of administration was subcutaneous and the site of inoculation was either side of the neck. A total of about 200 sera of single samples were collected and examined. All the animals were local indigenous zebu of both sexes and all ages.

Mycoplasma titre of vaccinal samples:

Two batches of T_1-SR freeze dried monovalent vaccine, PL17-94 and PL19-94 were collected from the Bahir-Dar and Dangila veterinary clinics and the probable number of mycoplasma particles was estimated. Although both vaccines were found with reduced titre as compared to the one they had during dispatching from the production laboratory, the minimum recommended organism per dose was conserved.

Metekel ranch:

A total of 60 animals (30 pure fogera and 30 Fresian/Fogera cross breed) were sampled since 55 days after vaccination and the sampling was followed on 84, 105 and 140 days post vaccination. The vaccine was applied by animal health professionals working in the particular ranch and it was known that all animals have received one ml of T_1-SR attenuated freeze dried mono-valent vaccine with a titre of $10^{8.04}$ CCU_{50} per dose, batch number PL19-94.

Andassa Ranch:

A total of 60 animals (30 pure fogera and 30 Fresian/Fogera cross breed) were used. 12 animals (six from each breed were used as non-vaccinated controls). Similarly, the vaccine was applied by animal health professionals working in the particular ranch and it was known that all animals have received one ml of T_1-SR attenuated freeze dried mono-valent vaccine with a titre of $10^{7.7}$ CCU_{50} per dose, batch number PL17-94.

Debre Zeit Research farm:

A total of 77 cross-breed cattle were used (27 unvaccinated controls and 50 vaccinated). The vaccine was applied by animal health professionals working in the particular farm and it was known that all animals have received one ml of the prepared vaccine. The vaccine used was T_1 -44, with a titre of $10^{7.8}$ CCU_{50} per dose, batch number of PL4 - 97. Serum samples were collected at an interval of a week, day 0, 7, 14, 21, 28, 35, 42, 49, 56 and 63. The samples were tested with both C-ELISA and CFT.

Results

Table 1: Prevalence of CBPP in 11districts of Awi and West Gojjam zones / North West Ethiopia /:

District	Villages	Samples	% CFT+ 1/10	% CFT+ 1/20	%ELISA+
Achefer	4	280	23.93	10.70	9.28
Ankesha	3	180	26.67	24.4	21.66
Bahir-Dar	5	280	25.71	15.30	12.10
Banja	2	80	66.25	66.25	26.00
Burie	2	160	21.89	16.87	5.60
Dangila	3	180	42.78	41.10	31.66
Denbecha	3	60	40.00	33.30	6.60
Guangua	4	480	12.50	6.87	8.10
Jabi-Tenan	3	140	15.00	10.70	2.85
Mecha	6	260	11.54	7.30	6.80
Sekela	3	160	15.00	9.30	5.00
Ranchs	2	120	9.17	5.8	10.00

The overall concordance achieved for the totality of field samples including vaccinated in ranchs is as follows:

		C-ELISA +	C-ELISA -	
CFT	+	120	322	442
	-	200	1738	1938
Total		320	2060	2380

Concordance = 78.1%

Table 2: The post-vaccination C-ELISA result of cattle at Metekel, Andassaa and Debre Zeit

sample origin	Days post-vacc	N0 Samples	mean % C-ELISA	N0 sera > 50% C-ELISA inhibition
Metekel	55	60	34	5
	84	60	38	6
	105	60	38	6
	140	60	23	0
Andassa	0	60	30	1
	50	60	38	7
Debre Zeit	0	77	38	0
	7	50	30	0
	14	50	45	15
	21	50	40	37
	28	50	24	0
	35	50	32	0

Note: Find the C-ELISA value for each vaccinated cow in the annex (Debre Zeit)

Serological response of dairy cattle (Debre Zeit diary farm) for T_1-44 strain and Prevalence and Incidence rates calculated in relation with CFT and C-ELISA values /Table3 & Graph 1/.

Table 3:

Days	0	7	14	21	28	35	42	49	56	63
No cows	50	50	50	50	50	50	50	50	50	50
N0 CFT + 1/10	0	5	9	26	45	20	1	3	1	0
N0 CFT + 1/20	0	5	9	20	8	20	0	0	0	0
Prevalence CFT 1/10	0	10%	18%	52%	90%	40%	2%	6%	2%	0%
Prevalence CFT 1/20	0	10%	18%	40%	16%	40%	0	0	0	0
Incidence CFT	0	10%	10%	34%	36%	2%	0%	0%	0%	0%
C-ELISA+	0	0	15	31	0	0	0	0	0	0
Preval. C-ELISA	0	0	30%	62%	0	0	0	0	0	0
Incid. C-ELISA	0	0	30%	34%	0	0	0	0	0	0

According to the experimental result using the T1 44 strain, it was observed that C-ELISA antibodies were detected only on samples at day 14 and 21 and none on samples at day 0, 7, and day 28 on wards. CFT antibodies however, were detected starting at day 7 and reach peak at day 28 and decline starting day 35 on as indicated on the table and graph below. Unvaccinated controls remained sero-negative in both C-ELISA and CFT during the study period. Although, the vaccine strains used at field and on station are different, the sero-conversion rate on vaccinated cattle in ranches, where some sera were positive before inoculation, is low on samples at day 50 (Andassa).

The concordance of CFT and C-ELISA calculated on experimentally vaccinated cows (Debre Zeit dairy farm)

	C-ELISA +	C-ELISA -	
CFT +	25	37	62
-	27	681	708
Total	52	718	770

Concordance = 91.7%

Comparison of C- ELISA and CFT:

The comparison was made on results of serum samples for CBPP epidemiological surveillance and for evaluation of the vaccinal humoral response. The CBPP sero-prevalence recorded by CFT in the surveyed districts varied from 5.8% on ranches to 66.25% (Banja) and by C-ELISA from 2.86% in Jabi-Tenan (vaccinated since 4 months) to 26.0% (Banja). For the totality of sera from the surveyed districts of Awi and western Gojjam, the CFT and C-ELISA sero-prevalence is 18.58% and 13.4% respectively.

The observation on experimentally inoculated cattle have shown that complement fixing antibodies appear early and reach a pick at days 21 to 35. At day 21 a higher proportion of immunised cows (62 %) were screened by the C-ELISA (mean competition value 57%) as compared with the 40% of the CFT. Although, the strains are different, the serological result achieved on field vaccination sera (day 55-140) is in full agreement with the experimental one. The presence of few positives may be due to endemic status of the sites.

APPENDIX 7

KARI
KENYA

SCIENTIFIC REPORT

Dr H. O. WESONGA

KENYA AGRICULTURAL RESEARCH INSTITUTE
NATIONAL VETERINARY RESEARCH CENTRE MUGUGA, P.O. BOX 32, KIKUYU, KENYA
TELEPHONE, KARURI 0154-32000, 32703, 32016, 32107, FAX: 0154 - 32450,
TELEGRAMS: VETRESORG; KIKUYU

When replying please quote

Our Ref. .....

Date.....

Dr. J.J. Tulasne
CBPP/EU Project coordinator
CIRAD-EMVT
BP 5035
34032 Montpellier
France

03/11/97

Dear Dr. Tulasne

Please find enclosed the statement of expenditure for workplan No. 2, the global statement of expenditure duly signed by our financial officer and the final end of year report with alterations, being sent by DHL. Receipts of the recent expenses are dated before October 17.
I should justify 4,655.13. As a result, I have included a receipt of a deposit for equipment which I have not yet paid for and which I look forward to receiving in the period of workplan 3. I hope this is acceptable.
Out of the ECU 4,655.13, you have sent 3,500. Please let me know if I should expect the difference soon. If available, I will pay the deposit for the shaker to fasten the delivery.
Best regards

H.O. Wesonga

END OF WORKPLAN 2 SCIENTIFIC REPORT, October 1997

EU/CBPP Project, Component 2

H.O. Wesonga
Kenya Agricultural Research Institute (KARI)

Like in the previous year, KARI participated in component 2 of the project in which contagious bovine pleuropneumonia/competition ELISA (CBPP cELISA) and complement fixation test (CFT) were carried out. Whereas CFT is an old test in our laboratory, cELISA was introduced during year one of the EU/CBPP project. Unlike in year 1 of the project, cELISA was carried out with less difficulties during year 2. This was mainly as a result of the visit by one of our staff to CIRAD-EMVT where they carried out effective practice and consultations. Meetings with the coordinators during the year, more particulrly with Dr. Thiaucourt, coordinator of component 2, also contributed to enhancing our perfomance.
Both cELISA and CFT were carried out on serum samples from the field as well as on those samples received from Namibia in year 1 of the project. When carried out on the same sample, a close relationship was observed between the results of both cELISA and CFT. Results of the samples tested at NVRC were submited to the coordinator of component 2 of the project. Some of these samples confirmed the presence of CBPP and formed a basis for the Department of Veterinary Services to institute disease control measures in affected areas. The work was carried out in two parts.

1. Collection and testing of serum samples.
In addition to the serum samples received from Namibia, samples were collected from CBPP as well as non-CBPP areas in Kenya. With the exception of Kilgoris, a foci of CBPP in Western Kenya, samples from CBPP areas were from suspected outbreaks in Eastern parts of Kenya while those from non-CBPP areas were from Western parts of Kenya. These samples were tested using CFT and cELISA. Samples from Namibia which had not been inactivated when carrying out CFT in year 1 of the project were also tested during the current year using both CFT and cELISA. The number of samples found positive out of the total number tested are shown below:
Namibia: 105/194.
Kenya: a) CBPP cases - Embakasi 17/26, Garissa - 6/13, Machakos - 26/100,
Kilgoris - 6/6, Thika(from various herds) - 7/70.
b) Non -CBPP areas: Eldoret - 1/25, Kakamega - 0/25.
All those samples that were positive on CFT were also positive on cELISA.

2. Collection and culture from lung samples.
Two slaughterhouses around Nairobi were identified as receiving animals from Eastern and North Eastern parts of the country where CBPP is present. Several visits were made to these slaughterhouses in search of lung samples with lesions suspected to be due to CBPP. Two cases were found, but on culture, the lungs were negative for *Mycoplasma mycoides* subsp. *mycoides (M.m. mycoides)*. Several lung samples were received from the field. One of the lung samples was received before the disease was known to be CBPP. This was from a postmortem case which had died following treatment for pneumonia. This lung was negative on culture for Mycoplasma. Following this report, a visit was made to Thika District where serum samples taken were found positive for antibodies to *M.m. mycoides*, on both cELISA and CFT (see above). Only six out of 70 cattle in the affected herd were found, the rest having either died or slaughtered due to the

disease. Slaughter of the six surviving animals revealed active as well as chronic lung lesions characteristic of CBPP. Culture from the lesions showed Mycoplasma colonies with smooth surfaces and a fried egg appearance. Growth inhibition test showed the following zones of inhibition (radius)

1. Rabbit anti-*M.m. mycoides* (SC), strain T1 - 3.9mm
2. Rabbit anti- *M.m. mycoides* (LC) strain F30 - 2.08mm
3. Rabbit anti-*M.c. capripneumoniae* - 0mm (no inhibition).

Arrangements are under way to send the lung samples and the isolate to CIRAD-EMVT and Uppsala for confirmation that it is *M.m. mycoides* (SC).

APPENDIX 8

LIRI
UGANDA

SCIENTIFIC REPORT

Dr E. TWINAMASIKO

Uganda: Annual Report 1997

Validation of C-ELISA for the diagnosis of CBPP: Progress Report

1. Introduction

Contagious Bovine pleuropneumonia (CBPP) made its reappearance in Uganda in the early eighties albeit in the North-Eastern part. It became a potential threat in the early nineties with the spread westwards eventually reaching Mbarara district in south western Uganda where the highest number of cattle are found. Currently CBPP is prevalent in almost all districts in Uganda. CBPP is considered as one of the most problematic diseases in the country because of its speed of dissemination, the uncertainty of diagnostic facilities, and insufficient knowledge/research on vaccines and postvaccination responses.

Initial attempts to control CBPP involved use of the universally accepted vaccine T_1-SR. The outcome has not been very satisfactory as the disease, initially diagnosed in Mbarara continued to spread to neighbouring districts. Uganda has now reverted to the original T_{1-44}.

Successful control of CBPP in Uganda (as anywhere else) will depend greatly on concerted effort in disease detection to remove infected animals and, serological monitoring to support vaccination programmes.

Disease detection is based on clinical observations must be backed by laboratory confirmation since CBPP may be confused with other respiratory syndromes.

Serological monitoring will offer a reliable system for monitoring the spread of the disease and the success of vaccination programmes.

Strong capacity for diagnosis and large scale testing of survey samples that employs a reliable test is therefore an important tool.

In the past we have employed the slide agglutination test, the Complement fixation test (CFT) and the indirect ELISA. None of these tests have been satisfactory in the detection of all the disease categories (i.e. the clinical, subclinical and chronic cases) of CBPP. The Complement fixation test continues to be the routinely used because the one recommended by OIE. The CFT does not detect chronic infections as are commonly found in endemic areas. It is also tedious and unsuitable for large scale testing.

Livestock Health Research Institute has participated in the CIRAD-EMVT coordinated validation of the competition ELISA and evaluate its potential for routine use in the diagnosis and monitoring of CBPP in Uganda.

2. Materials and methods

2.1 Samples

Samples for the validation exercise were taken from three distinct epidemiological categories. The first set of samples was taken from herd that had active cases of clinical CBPP, the second set of samples were from CBPP endemic areas. Samples were also taken from herds that had no CBPP in the last five years. All these herds had not been vaccinated in the last three years.

2.2 Serological tests

All samples were tested for antibodies to ***Mycoplasma mycoides sub-species mycoides*** SC using the competitive enzyme immuno-sorbent assay (C-ELISA) and the compliment fixation test. The

compliment fixation test (CFT) was carried out by the micro modification of the Campbell and Turner, 1953 [1] method, following the protocol recommended by Le Goff and Thiracourt [2]. The test protocol used for the C-ELISA was that proposed by CIRAD-EMVT [3] in the validation protocol for the CFT and C-ELISA test. A complement titration was also carried out to determine the complement end point titre,. A conjugate titration was carried out before the samples were tested

3. Results

3.1 Samples

The total number of samples collected are shown in table 1.

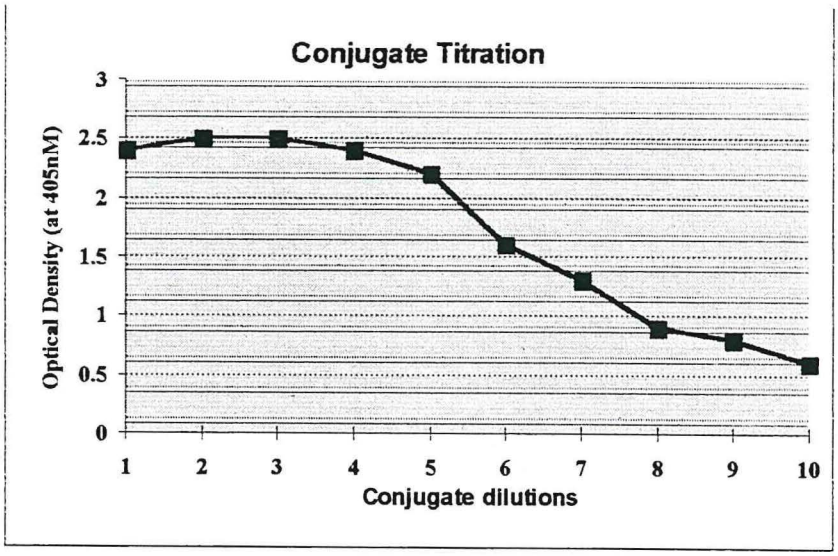
Table 1

Disease/vaccination status	District sampled	Number of samples
Active disease	Soroti	260
Clean,unvaccinated	Iganga,Apach	312
Endemic, unvacc	Kotido,Moroto	72
TOTAL		644

3.2 Conjugate titration

The conjugate titration curve is shown in figure 1.

Fig 1 Conjugate titration curve

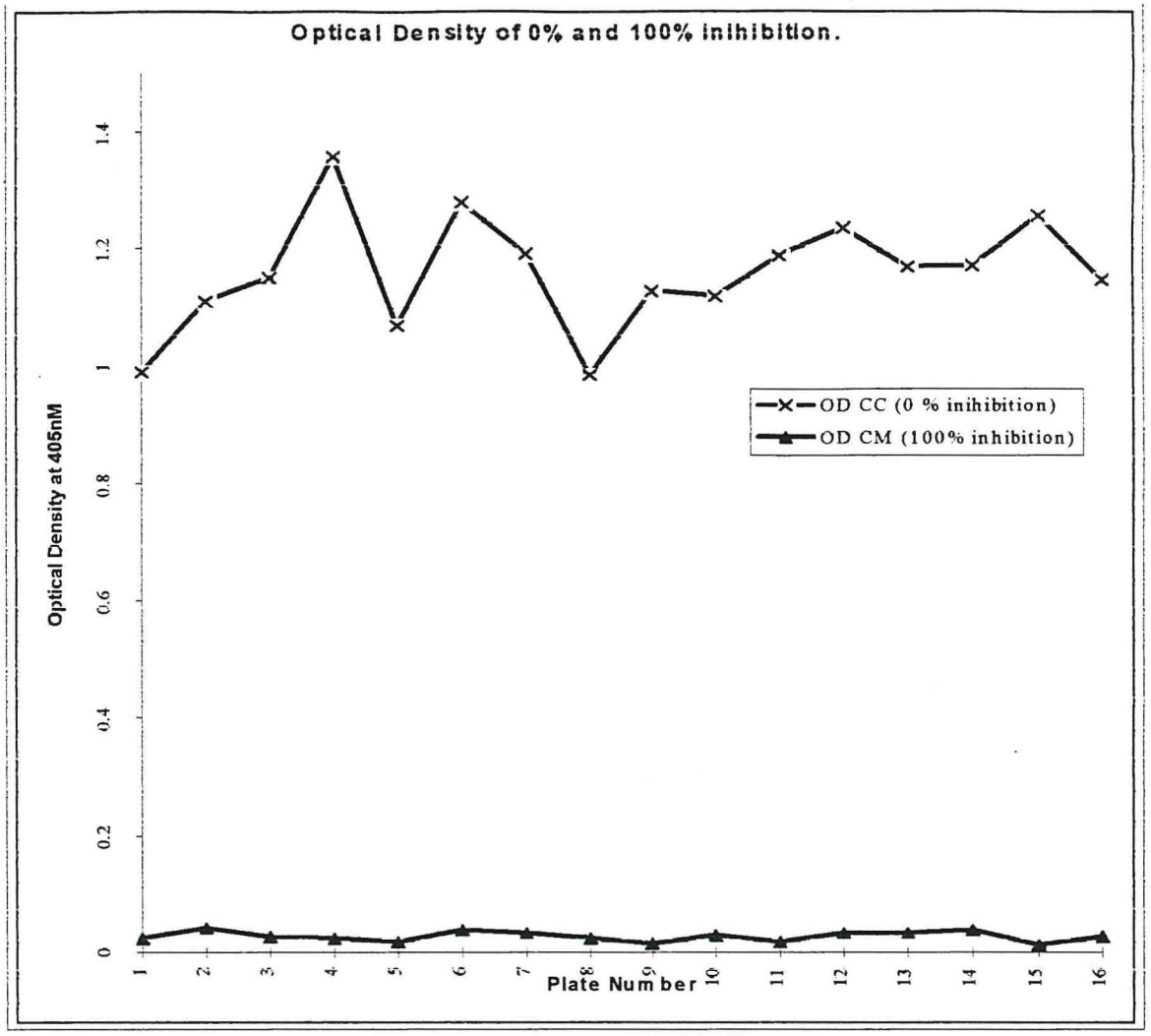


The complement end point titre of control sera was 1:1280.

The optical density of the conjugate control (0% inhibition) ranged from .012 to .040. The monoclonal control (100 % inhibition) OD ranged from .985 to 1.358. The OD values for the

conjugate and monoclonal controls for each test are shown in Figure 2.

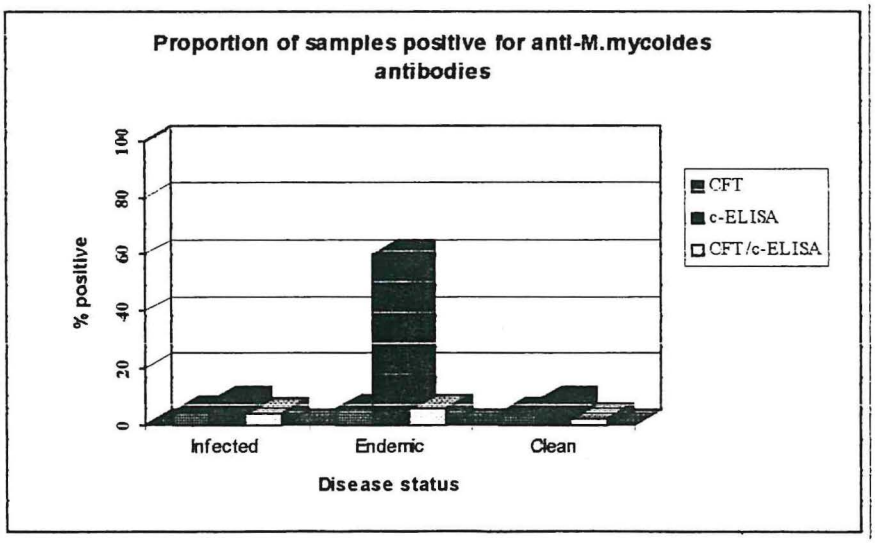
Figure 2



3.3 Antibody response

The proportion of samples positive for anti M. Mycoides antibodies for each of the tests used is shown in Figure 3.

Figure 3:



Most of the samples from the infected and the clean herds which were positive to CFT were also positive to ELISA (only 3 samples in each of the two were positive to CFT but not to ELISA). This discrepancy did not occur in the samples from herds in endemic areas.

4. Conclusion

The results indicate that the C-ELISA picked more positives in all cases, especially in the endemic herds. Although this seems to indicate that the C-ELISA may be a more sensitive test for detection of CBPP cases both in the new outbreaks and in the endemic areas, definite conclusion can only be made after follow up of this herds. This may however be difficult since some of the herd are nomadic and others are likely to have been slaughtered. The wide variation seen in the day to day reaction of the monoclonal control may be due to operator error and may disappear with experience. The reaction of the conjugate controls was fairy consistent.

5. References

[1] CAMPBELL AD, TURNER AW, (1953), Studies on Contagious Bovine Pleuropneumonia of cattle IV. An approved complement fixation test. Aust.Vet. J., **29:** 154-163.

[2] LE GOFF and THIRCOURT F, Validation protocol for CFT and C-ELISA, CIRAD-EMVT: Micromethod Complement Fixation Test for Contagious Bovine Pleuropneumonia. Contagious Bovine Pleuropneumonia Research programme : Annual Report year 1, Appendix 5.

[3] Validation protocol for CFT and C-ELISA, CIRAD-EMVT: Competitive enzyme immunoassay for detection of antibody to ***Mycoplasma mycoides subsp. mycoides SC***,: Contagious Bovine Pleuropneumonia Research programme : Annual Report year 1, Appendix 5.

APPENDIX 9

IZSAM
ITALY

SCIENTIFIC REPORT

Dr A.PINI

istituto zooprofilattico sperimentale
DELL'ABRUZZO E DEL MOLISE

SEDE: TERAMO: Via Campo Boario - Tel. (0861) 3321 - Fax (0861) 332251
Partita IVA: 00060330677 Cod. Fiscale: 80006470670

SETTORE

PROTOCOLLO:

CBPP/EU RESEARCH PROJECT N.5100.35.94.917
REPORT OF ACTIVITIES CARRIED OUT AT TERAMO INSTITUTE, YEAR 2

In the course of year 2, it has been agreed to transfer to CVL, Windhoek, Namibia, some of the funds and activities originally indicated in Components 1 and 3, of the Teramo budget and more specifically "challenge of cattle vaccinated with Iscom vaccine" and "related activities in Namibia" respectively. On the other hand funds allocated in Component 1 for "travel and accommodation in Namibia" were not utilized for this purpose because of the delays in conducting the Iscom vaccine experiment. Therefore the activities carried out by the Teramo institute during the year concerned Component 2: "diagnostic" and Component 3: immunology and pathology".

COMPONENT 2: Diagnostic

Modification of complement fixation test (CFT). The CFT officially recognized by the OIE, for the identification of antibody to MmmSC, is carried out according to the procedure described by Campbell and Turner. It requires 12 U of emolisin, 2.5 of C' and red blood cells (RBC) at a concentration of 6%.

This study has been carried out in an attempt to standardize CFT for CBPP with CFTs performed for other pathologies. Various concentrations of RBC were tested against different concentrations of emolisin and C' in cross check board titrations in presence and absence of antigen. Preliminary tests were performed with positive and negative standard sera. CFT according to Campbell and Turner was used as control. The test, optimized using a concentration of 2% RBC, 2U of emolisin and C', was then performed with positive reference sera (806, 845 and 840) kindly provided by Dr Regalla. On the basis of the results, indicating a correlation between the tests performed according to Campbell and Turner and the modified one, it was decided to carry out a comparative trial using 286 sera from the Institute serum bank.

The two tests have been compared using the Cohen Concordance Index. The concordance was equal to 0.96,whereas concordance beyond the chance was equal to 0.84. The results allow us to state that the two tests are interchangeable for the identification of positive and negative sera (see Table). The titres expressed in natural logarithm are significatively correlated (Pearson Correlation coefficient=0.89, $p<0.001$). Therefore the two tests are interchangeable for antibody titre determination. The modified test is easy to perform and has the advantage of saving reagents.

		TEST 2%			
		positive	negative	doubtbf.	Total
TEST 6%	positive	241	1	2	**244**
	negative	0	30	4	**34**
	doubtf.	4	1	3	**8**
	total	**245**	**32**	**9**	**286**

Evaluation of c-ELISA and Latex agglutination test using Teramo serum bank will be carried out once the kits will be received.

COMPONENT 3: Immunology and pathology

This work will provide additional information about the pathogenesis of the disease and could be an

This work will provide additional information about the pathogenesis of the disease and could be an additional tool in the evaluation of the Iscom vaccine by ascertaining whether immunized animals are protected not only from clinical disease from infection as well.

Samples of lungs and thoracic lymph-nodes were collected from 20 CBPP naturally infected cattle from Portugal and from 10 Italian animals in which CBPP-like lesions were observed at the abattoir. They were processed for histological, immunohistochemical and ultrastructural studies. Similarly, samples from nasal mucosa, thoracic lymph-nodes, tonsils, lungs, pleura and sinoviae, from 5 experimentally infected cattle from Cameroun (LANAVET), were collected and processed. Unfortunately, results of laboratory examinations of this latter group of specimens were inconclusive as they had not been collected and stored in appropriate way. Lymphocyte phenotypic characterization studies were not performed as frozen material was not available.

Histological and immunohistochemical studies

Histology: necrotic areas in sequestra, fibro-connective capsule, vessels and lymphoid foci in the pulmonar parenchima were studied. Peri- and vasculities with thrombi in the vessel lumen were evident. Thoracic lymph-nodes showed only intense hyperplasia.

Immunohistochemistry: a marked positivity to PAP (peroxisade-anti-peroxidase) and ABC (avidin-biotin complex) was observed in the alveolar and bronchiolar macrophages, and in few cells surrounding small and medium vessels. It is interesting to note that positive reactions were also evident in macrophages out of sequestra. A failure to block the infection could be at the basis of this finding, however further studies occur to prove it.

Typical diffuse reaction, as described by others, was seen in the lymph-nodes however, in some of them the reaction had a characteristic half-moon pattern. As far as we know, this is the first time that a similar pattern was described in CBPP lesions. A different follicular distribution of the positivity might be the expression of two different morphofunctional stages in the recognition and processation of *Mycoplasma*.

Ultrastructural studies

Methods to process samples for TEM (Trasmission Electron Microscope) were optimised and standardised. Samples were processed and embedded in Epon 812 and Lowicril resin for ultrastructural and immunocytochemical studies, respectively. Ultrathin sections from healthy animals were prepared and used as control. Specimens examination is under way.

Ultrastructural as well as immunocytochemical studies will be very useful in understanding mechanism at the basis of the pathogenicity of *MmmSC* and its interaction with the host.

Attilio Pini

Approved

Vincenzo Caporale

Director

APPENDIX 10

CIRAD-EMVT
FRANCE

SCIENTIFIC REPORT

Mrs V. BALCER
Dr L. DEDIEU

Contagious bovine pleuropneumonia :

Second annual scientific report

Second experimental reproduction of the disease :

Study of the cell-mediated immune responses

During the second year of the CBPP project, a second experimental infection was held in Garoua (Cameroon) in order to verify the results obtained from the first year of study and to get correlations between the immune responses described before and protection.

I) Experimental infection

The protocol followed for this second experimental infection was almost the same as for the first year. A first group of animals has been artificially infected by endobronchial intubation. A second group of zebus was used to study the natural infection.

For each animal:

- temperature and clinical signs have been recorded
- blood samples and bronchioalvelar lavages (BAL) were collected almost each week
- lymph nodes were taken when slaughtering the animals

A) Clinical results obtained for group 1 :

8 zebus were infected on may 12th and 13th 1997 with a fresh culture of the Afade strain of *Mycoplasma mycoides* subsp. *mycoides* S.C. (*Mmm*SC) (50 ml of 10^7 CFU/ml + 30 ml of 1,5% sterile agar) by endobronchial intubation in the right lung. 5 of these animals had a BAL preliminary to the intubation (I4, I5, I6, 855, 857).

I1; I2; I3; I4; I5; I6 were new zebus bought for the second experimental infection.

855 and **857** were the 2 zebus remained from the first year project, both were in the "in contact" group during the first experimental infection. None of them had developed any clinical symptoms and the serology had remained negative during all the study.

* First month post infection

I1 - *died of acute CBPP 3 weeks post infection (p.i.)*

857- *died 3 weeks p.i. without clinical signs of CBPP but typical lesions were found at the post mortem examination*

855- *had temperatures above 39°C from day 3 to day 15 p.i. , then developped mild clinical signs 2 weeks p.i. and recovered. This animal had a chronical form of CBPP . Severe bronchial lesions were observed with the bronchoscope.*

I2; I3; I4; I5; I6 *did not developed any clinical signs.*

*Boost performed one month p.i.

Due to the death of 2 animals and to the low pressure of infection of the remaining animals, a second intubation with a culture of the Afade strain was performed and one new animal was added (**I0**). The boost was given 4 weeks p.i., without any preliminary BAL.

I0; I2 and **I5** *presented an acute form 6 days after the boost and then a stationary state. Severe bronchial lesions were observed with the bronchoscope.*

I4 and I6 : *subacute form with mild bronchial lesions and then stationary state.*

I 3 : *no clinical signs*

All the animals, excepted **I3 and I6**, were slaughtered 5 months p.i..

The post mortem examination had demonstrated that all animals had classical lesions of CBPP in the right lung or at least sequestra. No lesion was observed in the left lung except for **I1** where pleural fluid was found.

All animals presented a positive serology in competitive ELISA and CFT.

B) Clinical results obtained for group 2 :

16 new zebus were put "in contact" with the zebus from group 1, 3 days after the experimental infection, without expecting any clinical signs (16.05.97).

The "in contact" zebus were named C1 to C16.

3 animals died in the first 3 months : **C1** of *an unknown reason*, **C11** of pericarditis and **C14**, with no clinical sign but the post mortem examination revealed several liters of fluid with big pieces of fibrinous material in all the thoracic cavity and strong adherence with pleura.

6 animals died or were slaughtered due to respiratory distress, 2 to 3 months post contact. All had typical CBPP lesions in one or both lungs.

C7 : slaughtered the 14.08 due to respiratory distress
PM : typical CBPP lesions + pleural fluid in the right lung and beginning in the left lung.

C8 : dead on the 14.07;
PM : typical CBPP lesions + pleural fluid in both lungs

C9 : slaughtered the 14.07 due to respiratory distress
PM : right side affected, adherence, 2/3 of the lung hepatised, no thoracic fluid.

C12 : mild clinical signs and death on the 03.08 ;
PM : typical CBPP lesions + pleural fluid in the left lung

C13 : slaughtered the 08.08 due to respiratory distress
PM : typical CBPP lesions + pleural fluid in the right lung

C15 : mild clinical signs. slaughtered the 25.08
PM : sequestra of 25cm + CBPP lesions in the right lung.

All the remaining animals had no clinical signs or very mild and were slaughtered 4,5 months post contact.

C2 : *PM : characteristic chronic form of CBPP with only left side*

affected with a melon size sequestrum.

C3; C4; C5; C6 *were negative at the post mortem examination*

C10 : *PM : characteristic chronic form of CBPP with a melon size sequestrum observed in the right thoracic part of the lung.*

C16 : *accidental death on the 29.09.97. PM : sequestrum on intermediary right lobe, small adherence with pleura.*

Among the 15 "in contact" animals, 13 became positive in competitive ELISA and CFT. Seroconversion occured from week 5 to week 14 after contact. C11 and C12 remained negative.

C) Clinical results obtained for the control group :

2 zebus were used as control (T3 and T4).

Both received an endobronchial intubation of sterile culture medium containing agar and T3 had a BAL preliminary to the intubation.

These animals did not developed any clinical sign.

II) Cellular Immunological studies

For each animal, a phenotypic "ex vivo" study is carried out by flow cytometry in order to follow the evolution of the various cell populations in the blood and in the BAL. Furthermore, the responsiveness of lymphocytes to *Mmm*SC is assessed by functional assays *in vitro*. All the techniques used in this study were already described in the previous scientific reports .

A) Phenotypic characterization of the blood cells

Group 1 : artificially infected animals
All the kinetics are presented in annexe.
Group 2 : phenotypic studies are underway;

B) Studies of the bronchoalveolar lavages (BAL)

Results of the phenotypic study of the BAL cells are not yet available since we are facing a lot of technical problems when studying these cells by flow cytometry.

No functional studies can be performed with these BAL since, the number of cells contained in each sample is too low.

C) Lymphoproliferation tests (LP tests)

All the "*in vitro*" stimulation were performed with
- a negative control (cells alone)
- a positive control (cells incubated with a mitogen : concanavaline A = ConA; 2,5 µg/ml)
- a solution of heat-killed *Mmm*SC (5µg/ml)

Since, as for the previous experimental infection, a lot of samples contained already dead cells, a preliminary test was performed to select viable samples.

The samples taken from each animal are listed below, those in bold correspond to viable

samples.

These samples were first tested by a colorimetric assay (MTS lymphoproliferative assay) and by flow cytometry (following only the size and granulosity of the cells) to evaluate those responding to the heat-killed *Mmm*SC antigen. For those which were positive, a phenotypic study of the responding cells was or will be performed to identify the subpopulation of lymphocytes involved in the immune response.

C.1 : Results of the LP tests for animals of group 1

I1 (acute case)

4 samples were taken before the animal died :

- before infection
- **1,5 weeks post infection (wpi)**
- 2 weeks p.i.
- **3 weeks p.i.**

Only 2 samples (1,5 wpi and 3 wpi) were available for the LP tests since the remaining ones were contaminated.

The lymphoproliferation tests performed with the PBMC taken 1,5 wpi demonstrated that *Mmm*SC-sensitized lymphocytes are present in the circulating blood of this animal. 10% of the lymphocytes had proliferated when put in contact with the heat-killed *Mmm*SC.

The cells stimulated by *Mmm*SC were mainly CD4 helper-T-cells but some gamma-delta T-cells were also activated.

The same test performed on PBMC 3 wpi gave identical results with 17% of proliferating cells. But in that case, T-cells had proliferated but their state of activation was lower.

No results were obtained with lymph nodes since they were contaminated.

857 (acute case)

No result were obtained with any of the samples included the 2 lymph nodes since all cells were already dead.

855 (chronic case)

1 sample was taken before infection (03.03)

samples taken p.i.:

- **1 week p.i. (21.05)**
- **2 w. (26.05) +++**
- **3 w. (01.06) +++**
- **4 w. (10.06)**
- **5 w. (18.06)**
- **6 w. (23.06)**
- **7 w. (02.07)**
- **8 w. (08.07)**
- **9 w. (15.07)**
- **10 w. (22.07)**
- 11 w. (30.07)
- 13 w. (12.08)
- **15 w. (26.08) +**
- 16,5 w. (03.09)
- **17,5 w. (10.09) +**
- **18,5 w .(22.09) +**
- **20 w. (01.10)**

- lymph nodes - **mediastinal**
  - tracheobronchic

25 % of samples were not usefull for the lymphoproliferation assays. Among the samples tested, lymphoproliferation with *Mmm*SC was observed with high level only for the samples taken

2 and 3 weeks p.i. No stimulation was detected until 15 weeks p.i., where some *Mmm*SC-specific T-cells were circulating. This could be due to contact with other animals and mainly with those excreting the mycoplasma.

Therefore, *Mmm*SC-specific T-cells can be found in the blood of this animal mainly at the begining of the infection.

Lymph nodes should be the real source of these *Mmm*SC-specifc memory T-cells. Unfortunately, the tracheobronchic lymph node is contaminated and the mediastinal was weakly stimulated by the mitogen (i.e. high proportion of dead cells) and did not reacted with *Mmm*SC.

The phenotypic study of the responding samples demonstrated that a high percentage of T-cells were activated by *Mmm*SC since their level of interleukin-2 receptor (IL-2Rc) was higher than when incubated alone. Furthermore a higher level of MHC class II molecules was detected. Those T-cells corresponded for a high percentage to CD4 T-cells and for a lower one to gamma-delta T-cells.

Therefore, only 2 samples are available for the next step which should be the identification of the proteins involved in this specific immune response. Unfortunately very few vials of each sample are available to perform this study.

I0

2 samples were taken before infection (16.05; 09.06)
samples taken p.i.:

- 1 wpi (16.06)
- **1,5 wpi (20.06)**
- 3 wpi (30.06)
- **4 wpi (08.07)**
- **5 wpi (14.07)**
- **6 wpi (21.07)** +?
- **7 wpi (28.07)**
- **8,5 wpi (08.08)**
- 10,5 wpi (22.08)
- 12 wpi (04.09)
- 13 wpi (10.09)
- **14 wpi (18.09)** +++
- **16 wpi (30.09)** +
- lymph nodes - **mediastinal**
  - **tracheobronchic** +

47% of these samples contained cells that were already dead and therefore cannot be used for the LP tests. Among the others, a weak proliferation was obtained with the PBMC taken 6 wpi and 16 wpi and with cells from the tracheobronchic lymph node. Unfortunately, only one sample (14 wpi) presented a high lymphoproliferative response with *Mmm*SC. The phenotypic characterisation of the responding cells was not yet carried out.

I3

2 samples were taken before infection (11.04; **11.05)**
samples taken p.i.:

- **1 week p.i. (21.05)**
- **2 w. (27.05)**
- 3 w. (02.06)
- 4 w. (09.06)
- 5 w.= 1 week post boost (wpb) (17.06)
- 6 w. = 2 wpb (23.06)
- 7 w. = 3 wpb (30.06)
- 8 w. = 4 wpb (08.07)
- 11 w. = 7 wpb (29.07)
- 13 w . = 9 wpb (11.08)
- 15 w. = 11 wpb (25.08)
- 16,5 w. = 12,5 wpb (05.09)
- 17,5 w. = 13,5 wpb (11.09)
- **18,5 w. = 14,5 wpb (18.09)**

- **9 w. = 5 wpb (14.07)**
- 10 w. = 6 wpb (21.07)

70% of these samples contained cells that were already dead or contaminated and therefore cannot be used for the LP tests. None of the remaining one presented any stimulation with *Mmm*SC.

I3 is still alive.

I4

2 samples were taken before infection (**14.04; 13.05**)

samples taken p.i.:

- 1 week p.i. (21.05) Non testé
- **2 w. (27.05)** ++
- 3 w. (03.06) Non testé
- 4 w. (10.06)
- 5 w.= 1 week post boost (wpb) (17.06)
- **6 w. = 2 wpb (23.06)** ++
- 7 w. = 3 wpb (01.07)
- **8 w. = 4 wpb (08.07)**
- **9 w. = 5 wpb (15.07)**
- **10 w. = 6 wpb (22.07)** +++
- 11 w. = 7 wpb (29.07)
- **13 w . = 9 wpb (11.08)**
- **15 w. = 11 wpb (25.08)** +
- **16,5 w. = 12,5 wpb (05.09)**
- **17,5 w. = 13,5 wpb (11.09)**
- 18,5 w. = 14,5 wpb (19.09)
- **20 w. = 16 wpb (02.10)** +
- lymph nodes - **mediastinal** ++
- **tracheobronchique**

37% of the PBMC samples taken from this animal contained dead or contaminated cells. The LP tests demonstrated that some *Mmm*SC-proliferating cells were observed in the circulating blood at various time p.i. and then in one of the 2 lymph nodes but at a low level.

The phenotypic study of the responding cells is underway but the number of vials corresponding to the positive samples are very limited. Therefore, characterisation of the protein(s) involved in the specific activation of T-cells will not be possible.

I5

3 samples were taken before infection (14.04; 09.05; 12.05)

samples taken p.i.:

- **1 week p.i. (21.05)**
- **2 w. (27.05)**
- 3 w. (03.06)
- 4 w. (10.06)
- **5 w.= 1 week post boost (wpb) (17.06)**
- 6 w. = 2 wpb (23.06)
- 7 w. = 3 wpb (01.07)
- 8 w. = 4 wpb (08.07)
- **9 w. = 5 wpb (15.07)**
- 10 w. = 6 wpb (22.07)
- 11 w. = 7 wpb (28.07)
- **13 w . = 9 wpb (12.08)**
- **15 w. = 11 wpb (26.08)**
- 16,5 w. = 12,5 wpb (05.09)
- **17,5 w. = 13,5 wpb (12.09)**
- 18,5 w. = 14,5 wpb (19.09)
- 20 w. = 16 wpb (01.10)
- lymph nodes - mediastinal
- **tracheobronchique**

65 % of the samples taken from this animal contained dead or contaminated cells. None of the remaining samples tested in LP assays demonstrated any proliferation with *Mmm*SC.

I2 and I6

These 2 zebus have not been tested at that time.

C.2 : Results of the LP tests for animals of group 2

5 "in contact" animals have been tested (C7, C8, C9, C12, C13). Unfortunately, for these 5 zebus, all the samples were contaminated!!!!!

D) ***Cytokine study***

In order to define which subpopulation of CD4 T-cells is involved in the immune response, the cytokines secreted have to identified.

Detection and quantification of interferon gamma (IFNγ) secreted in the supernatant of each positive LP tests are now underway, since an ELISA specific for bovine IFNγ is available. In contrast, no equivalent assay is available for any other bovine cytokines. Therefore, they will be studied by bioassays which will soon be developed in the lab.

E) Selection of protein(s) involved in the immune response

The first step will be performed with 3 different fractions of *Mmm*SC proteins separated according to their molecular weight. For this study, proteins were first separated by SDS-PAGE electrophoresis and then transfered to nitrocellulose membrane by western blot. Nitrocellulose was then cut in 3 fragments corresponding to proteins of molecular weight > 50 KDa, those of a molecular weight between 50 and 30 KDa and those < 30 KDa. Each nitrocellulose fragment was then cut in small pieces, dissolved and sterilised by incubation in DMSO. The nitrocellulose was then precipitated in small particules by adding an aqueous solution (bicarbonate buffer). After washing, these suspensions will be divided in small aliquot and used in lymphoproliferation tests. By comparing the results obtained with *Mmm*SC (containing all the proteins) and with the 3 different fractions of proteins, a first selection of the proteins involved will be achieved.

Due to the fact that very few vials are available for each positive samples, this technique is first tested with other bacteria and other cells that are available in the lab.

This technique could be used when lymphoproliferation is detected using the colorimetric assay but the nitrocellulose particules hampered the use of flow cytometry to identify the reacting cells. Therefore, in order to eliminate those particules, an electroelution technique is also tested to extract the proteins of each fraction before the LP tests.

The PATHOTROP lab will soon be equiped with a liquid chromatography system, which will be used to prepare the proteins.

III) Conclusion

The conclusion of this second year of work will be, unfortunately, almost the same as last year.

The results obtained from the experimental infections clearly demonstrated that the protocol which was followed was a good one. Among the 9 artificially infected animals, 2 developped an acute form of CBPP, 6 presented a chronic form and one seemed to be resistant. This correspond to the repartition of the clinical form of CBPP already described. Among the 16 zebus put in

contact with the first group of animals, 10 have really been infected with *Mmm*SC, since they all had typical post mortem signs of CBPP. The incubation period varied from 40 to 75 days post contact. Among these 10 animals all the various clinical form of CBPP were observed. All these results demonstrate that this second experimental infection was successfull and this is a positive result since CBPP is not a disease easy to reproduce.

However, even if the study of the immune responses obtained from artificially infected animals is interesting, our aim is mainly to study the reactions of naturally infected animals. Unfortunately, even if we succeed in having all the various clinical form of natural CBPP, the immune responses could not be studied since among the 5 "in contact" zebus tested, all the samples were contaminated. Therefore the only available results correspond to some artificially infected animals. But even for those zebus, the number of samples lost (cells dead or contaminated samples) is very high.

Therefore we are facing the same problems as last year which have to be solved before begining the third study in Cameroon. It is clear that the main problems concerned the training of technician(s) and the equipment of the lab used to prepare and conserve in the best way all the samples. To overcome some of these problems: a technician from the Lanavet came at Montpellier for 2 months to be trained and the Lanavet will soon be equiped with a refrigerated centrifuge. The remaining ones, concerning the contamination and the conservation of the samples, should be solved by verifying the laminar flow and be sure that the electric alimentation of the -80°C remained constant.

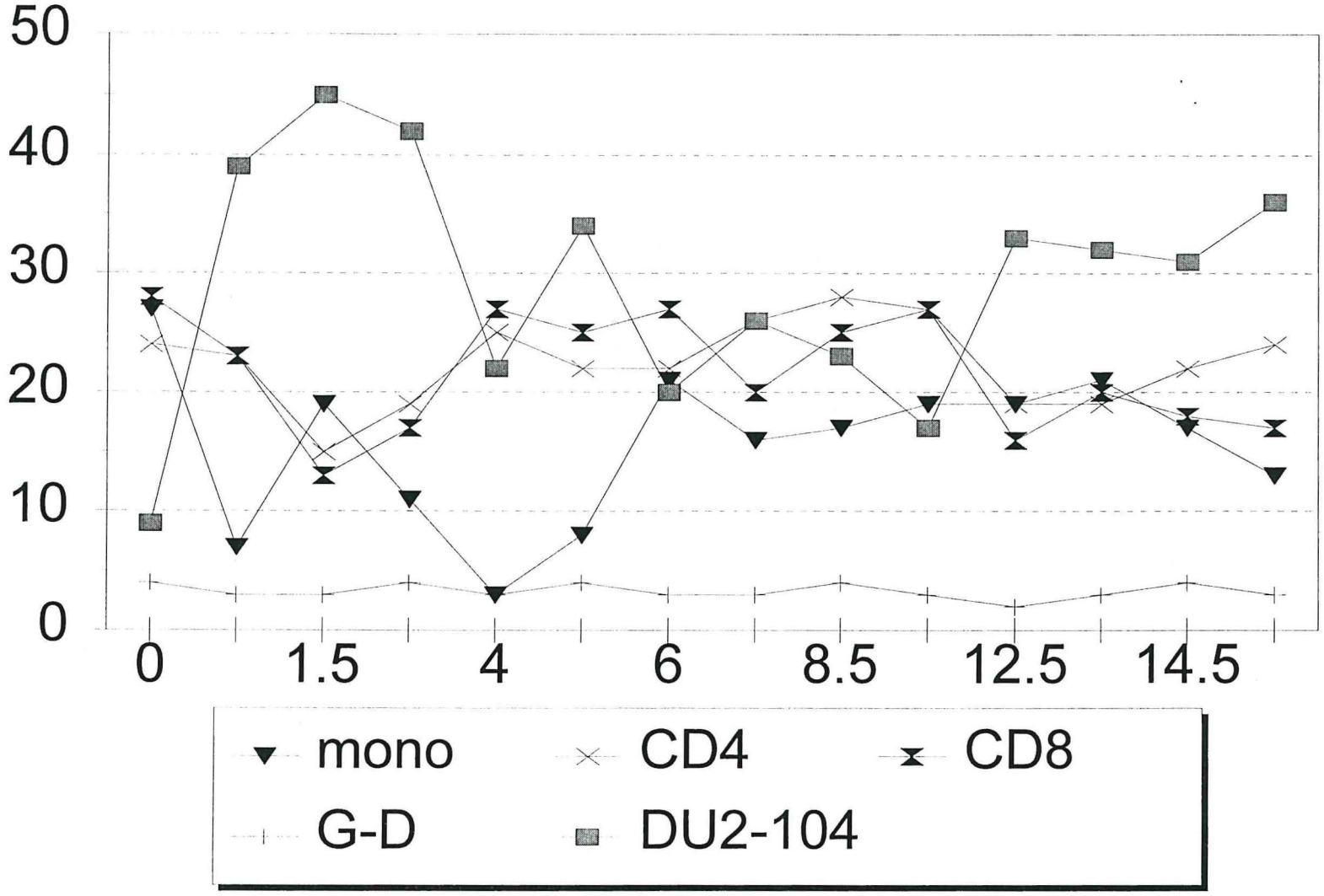
Concerning our scientific results, a lot of studies are still underway, but we can confirm that the *Mmm*SC-specific immune response of CBPP infected cattle is mainly based on CD4 T-cells. We cannot determine at that time the balance between the 2 subpopulations of CD4 : TH1 and TH2. The result of the IFNg ELISA and the development of a specific IL-4 bioassay will give this evaluation, unless the responding lymphocytes are from the TH0 group (i.e. secreting both IFNg and IL-4). Other interleukin-specific assays will help to characterize better the immune responses and to understand the immunopathology of CBPP.

The aim of this study is also to select the specific proteins of *Mmm*SC involved in the immune reaction and mainly those responsible for long- term protection. It is clear that to achieve these results, we need more viable samples of responding cells, mainly from lymph nodes, in order to be able to check fractionnated proteins.

In conclusion, we can say that we are able to reproduce the disease, artificially and naturally, since the two experimental infections were successfull, the MTS assay and the flow cytometry technique are now in routine use at the lab and give good results, various immunological assays will be developped to check the different cytokines and the fractionnated proteins will be ready to be tested, therefore to pursue this project, it is clear that we have to solve all the technical problems concerning the collection and conservation of the samples in order to get viable samples.

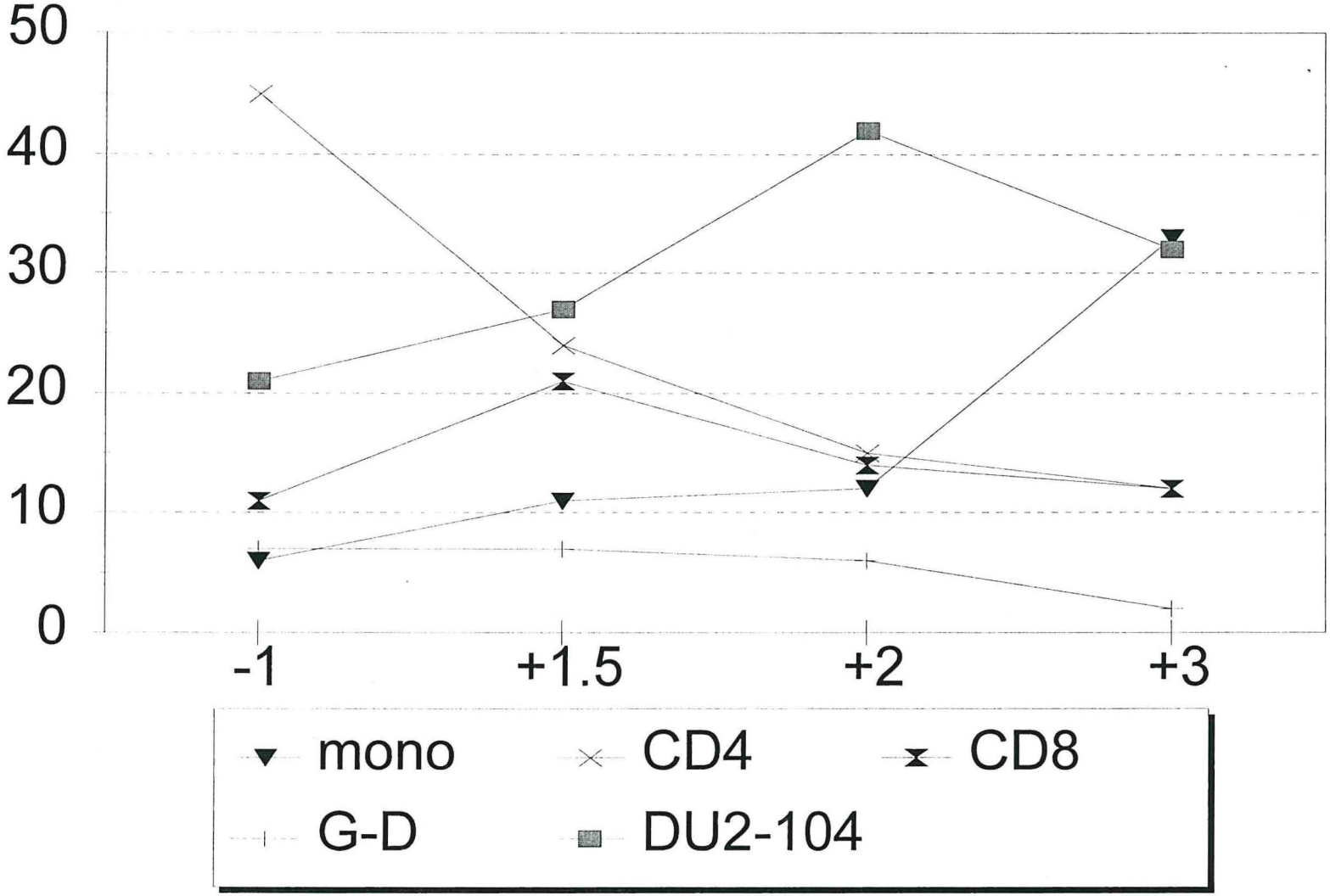
ZEBU I0 ex vivo

Infection le 10.6.97



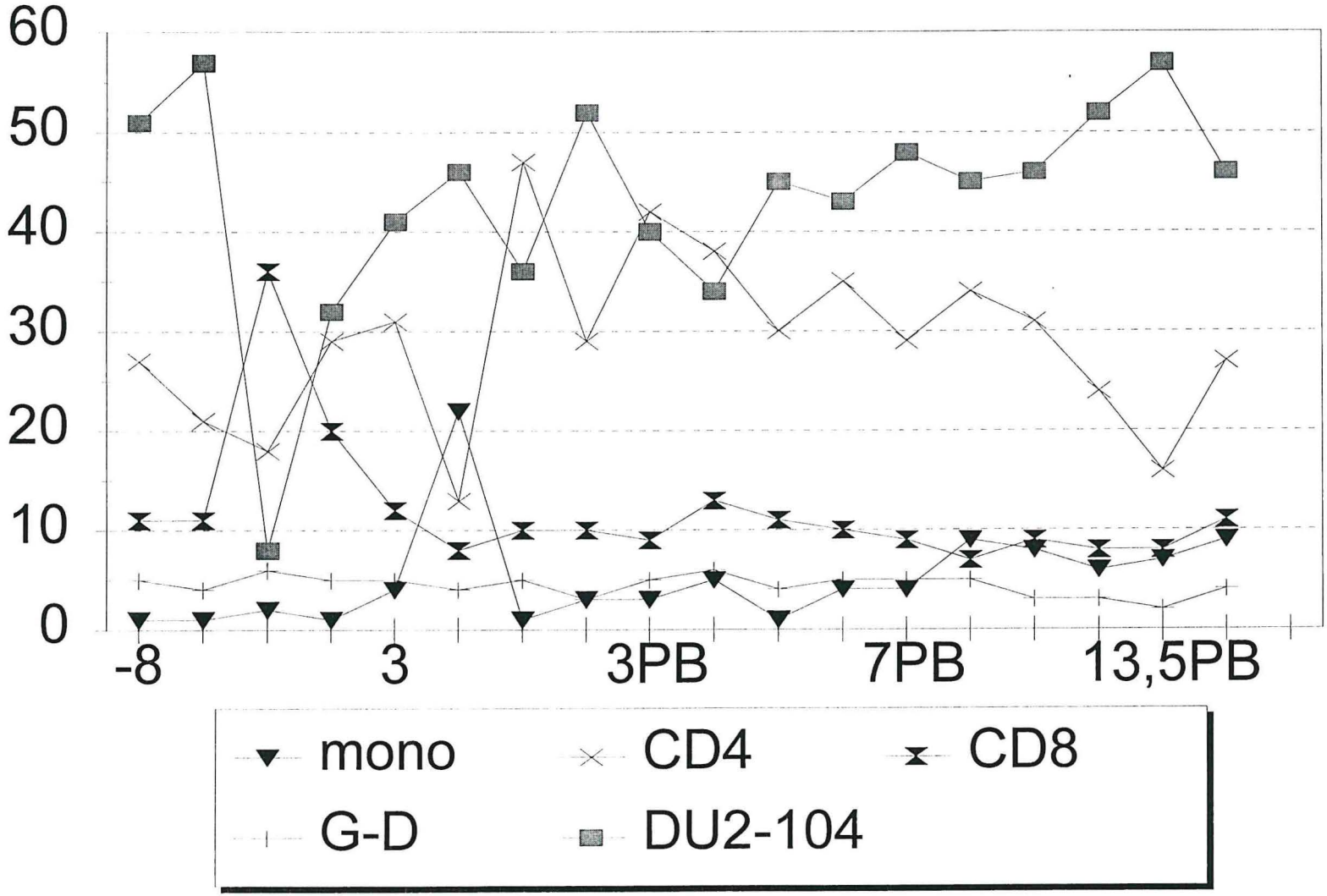
ZEBU I1 ex vivo

Infection le 12.5.97



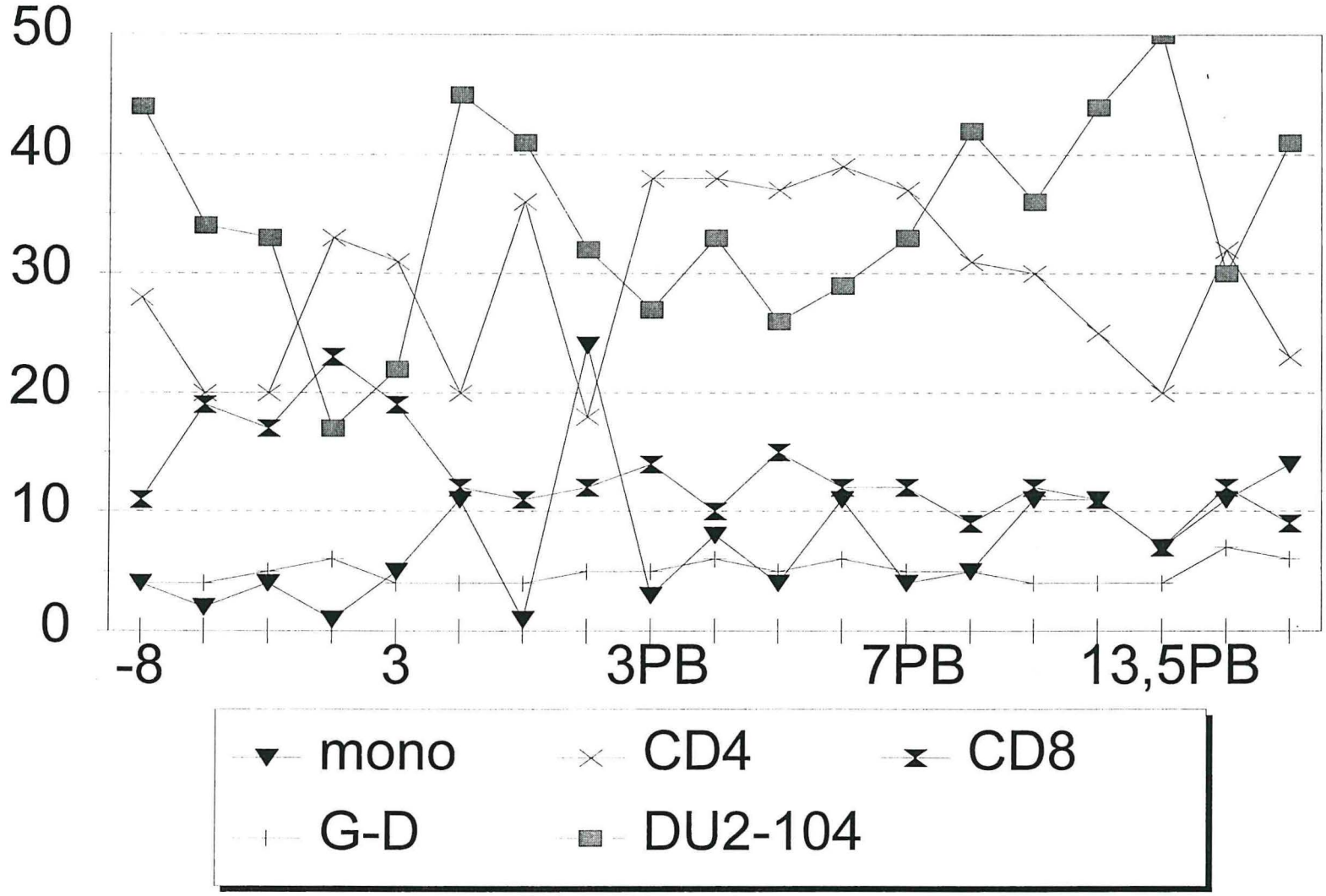
ZEBU I3 ex vivo

Infection le 13.5 + boost le10.6.97



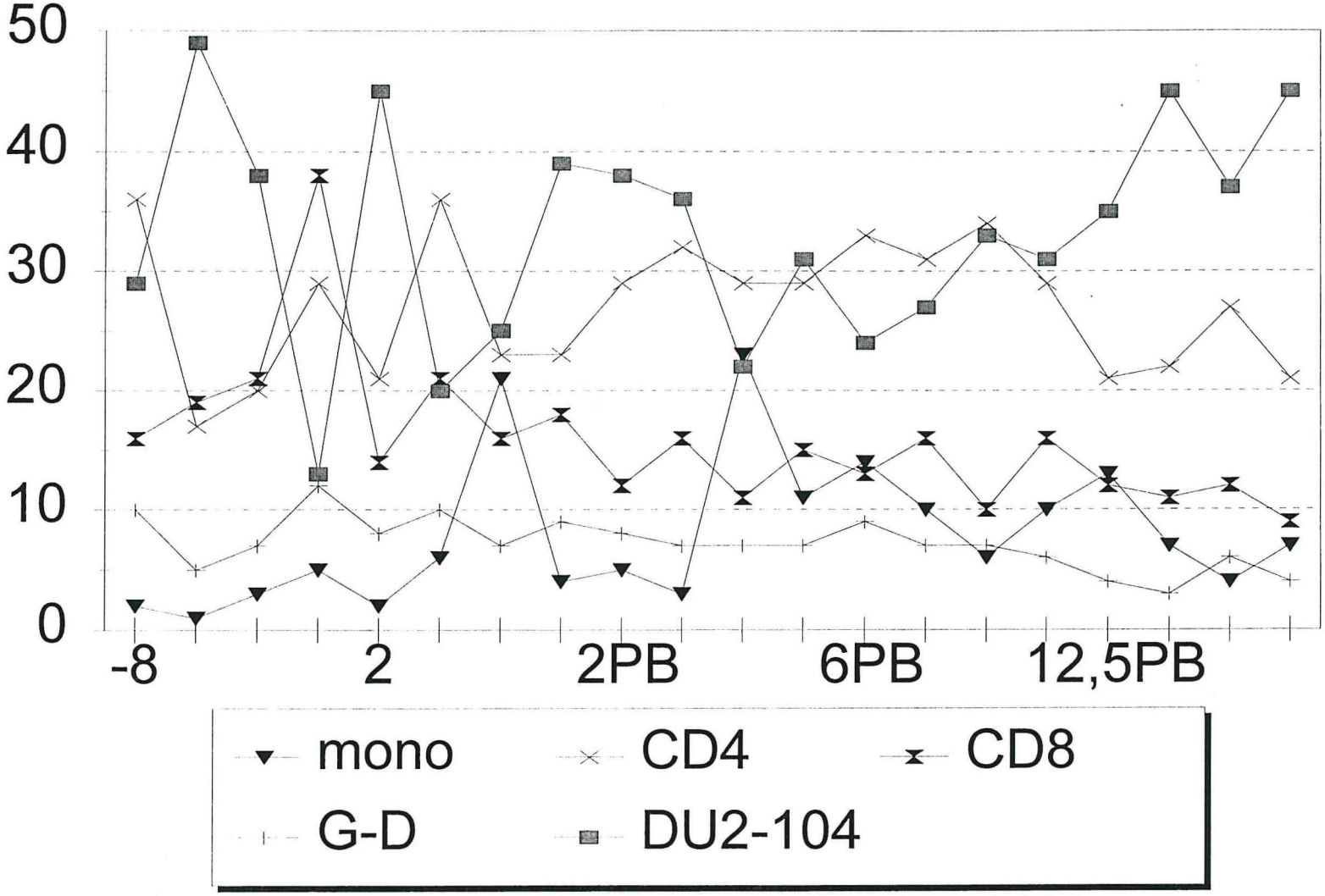
ZEBU I4 ex vivo

Infection le 13.5 + boost le10.6.97



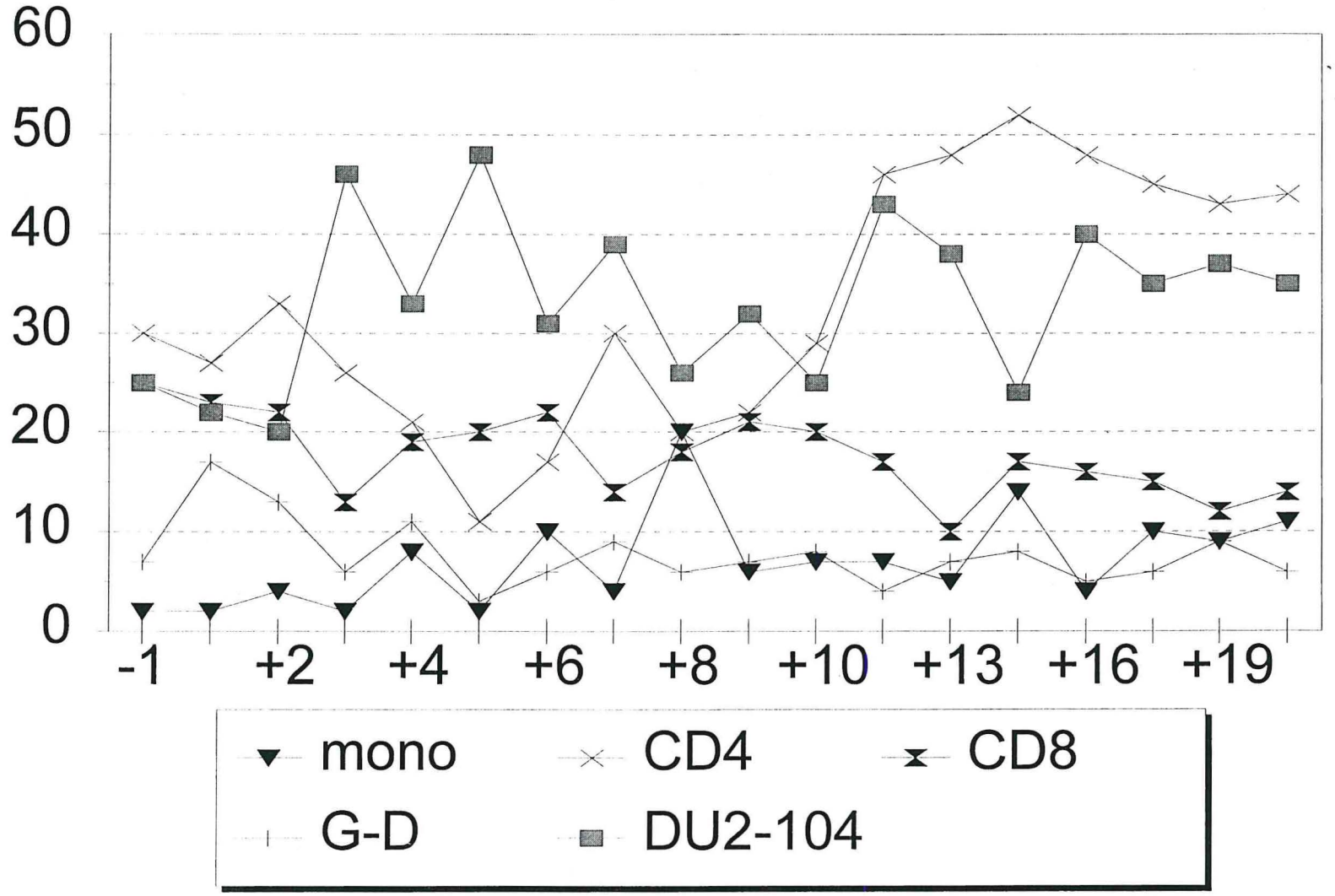
ZEBU I5 ex vivo

Infection le 13.5 + boost le10.6.97



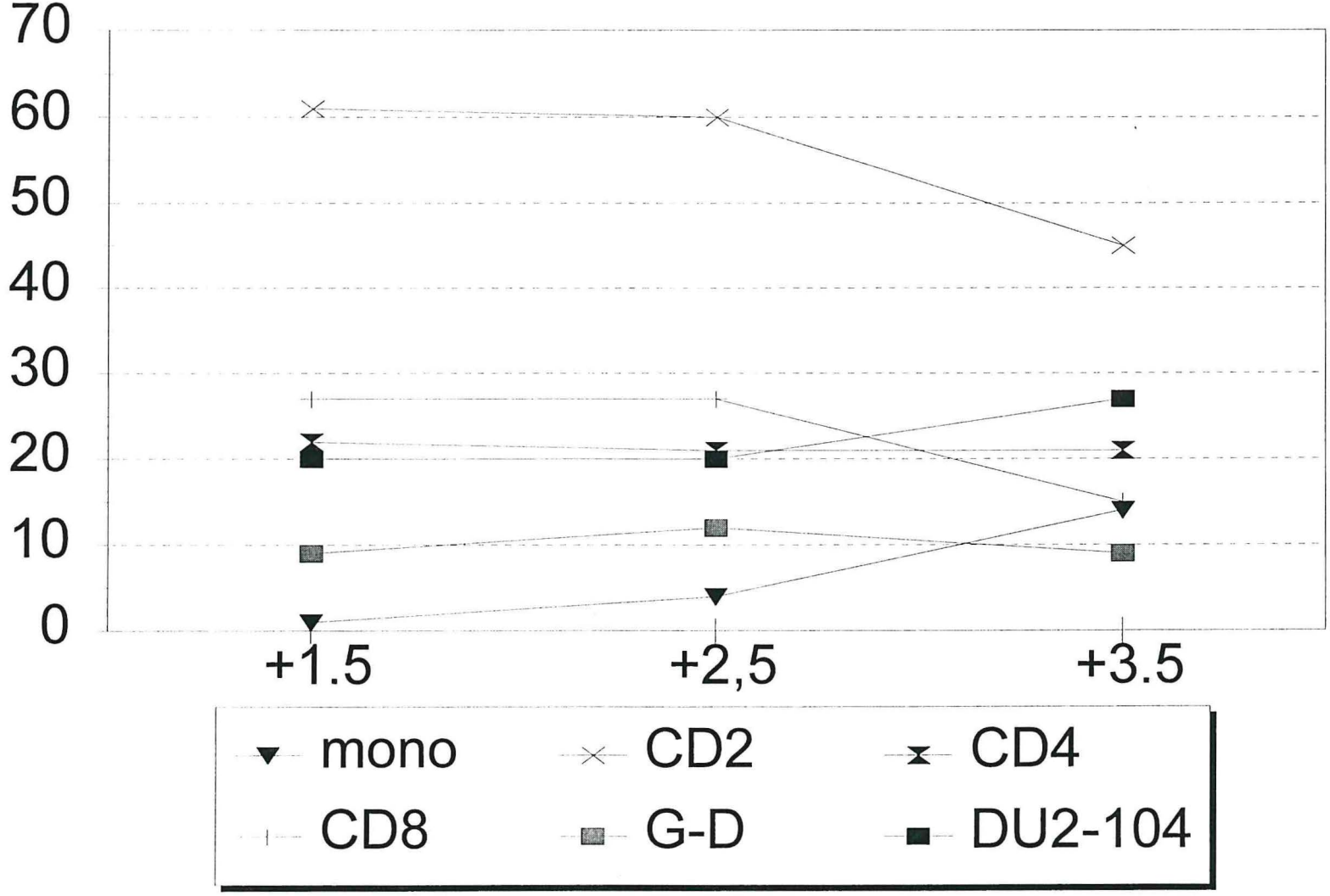
ZEBU 855 ex vivo

Infection le 12.5.97



ZEBU 857 ex vivo

Infection le 13.5.97



APPENDIX 11

LANAVET - GAROUA CAMEROON

SCIENTIFIC REPORT

Dr J. LOPES DE LIMA

CONTAGIOUS BOVINE PLEUROPNEUMONIA RESEARCH COMPONENT 3- IMMUNOLOGY

annual report : february-october 1997

Joachim lopes de lima
LANAVET
B.P. 503 Garoua
Cameroun

INTRODUCTION

The aim of the project is to improve understanding of immunological mechanisms leading to a state of CBPP disease or to immunity.
Since CBPP is primarily an evolutive disease localised in the lungs of infected animals, experiment will be designed to study local immunity processes in a dynamic way.
So the immune cells contained in Broncho Alveolar Lavages are specially studding as well as the cell populations contained in the blood. They are collected from experimentally infected and control animals before and during the course of infection.
The responsiveness of the lymphocytes and macrophages to MmmSC will be assessed by functional assays in vitro. With more accurate knowledge of the disease, it will be possible to assess whether existing vaccines against CBPP can be improved.

1- MATERIALS AND METHODS.

1-1: Selection of animals.

Over 3-years-old zebus are used throughout the experiment, 20 males and 5 females. Homogenous population (sex ratio, age) is impossible to find because most of the animals of North Cameroon are vaccinated against CBPP, and the protocol needs non vaccinated animals which are very difficult to find.
They health status is carefully control : complete clinical exam, brucellosis, tuberculosis, CBPP tested. Three of them are eliminated because of brucellosis.
As dry season occurred it wasn't very easy to find non- skinny animals. All of them are correctly identified by earrings.

1-2 : .Animals preparation

After carrying -out the cattle in LANAVET all animals are treated against worm- diseases with Ivomec-D, ticks with Bayticol, Trypanosomia with Trypamidium. All males are castrated to make them more comfortable to work with.
The population is then housed in two different places, one for the infected and contact animals, the other one (150 meters from the first) for control animals.
Infected animals are called I1 to I6 and are mixed with n° 855 and n° 857 which are 2 healthy animals contact from Hermann Hunger experiment. Contact animals are named C1 to C15 (15 zebus), and controls' T1 to T4 (4 zebus, in fact only 2 because T1 became C16 and T2 became I0 as we needed them for infection).
All of them are trained to enter in a restrain area every day, so that animals get use to be manipulated.
Their alimentation is made of grass (in wet season) and agroindustrial by- product with salt adjunction.

1-3 : **Experimental infection.**

The 8 zebus chosen for infection are infected with MmmSC on June 12Th and 13Th 1997. The inoculum is made of a 50 ml culture of Afade growing strain at 10^7 CFU/ml + 30 ml of 1,5 % sterile Agar (HBI dilution). All the group is inoculated by bronchoscope. After inoculation, 100ml of sterile culture is added.
I1,I2,I3 are infected with no BAL before. I4,I5,I6,855,857 with BAL before, so that the influence of BAL in CBPP development can be investigated.
Three days after infection, contact and infected animals are mixed together.
As only 855 get hill, and 857 with I1 died of acute CBPP, it's decided to make a second infection one month later with a 10^8 CFU/ml Afade strain and no BAL before inoculation.
To fill secure for this new infection, T2 is moved from its area and infected as well, it become I0.
On the other hand, control animals are managed as the former, excepted the inoculum is sterile. T3 has a BAL before inoculation, T4 hasn't. T1 is finally removed on C16 as C11 died quickly of an acute unknown pericarditis.

1-4 : **Immune cells collect.**

1-41 : Bronchoscopy technique.

A bronchoscope ETM 75 (13 mm diameter; 1,75 m long) is used, on one hand for inoculation and on the other hand for collecting lymphoid cells and liquids from broncho-alveolar lavages. It performs a good study of local immunity in a dynamic way and very interesting pictures of CBPP lesions which are helpful in the following of the disease. Also it allows to make BALs and inoculations in the area we decided to work with, it's a real advantage.

Animals are on diet for 24 hours before performing. They are then moved into a restrain corridor to maintain the zebu into an up position. At the beginning of the experiment a soft tranquillisation is used (0,5 ml xylazine at 2 %) to control the technique and to make the animals get use to it. Then chemical restrain is removed to allow fast recovery (specially when respiratory distress). In our opinion, it is necessary to work close to physiological conditions.

The nasal cavity is the entrance of choice for the instrument which is gently pushed to the larynx following the turbinate structures, once nostril cleaned with paper. When the nostril is to small, the technique is to pass through oral cavity using a PVC tube to protect instrument from destruction. In front of the larynx the introduction is stopped and the expiration time is waiting to move forward between the two arytenoid cartilages. Then trachea can be followed until bifurcation, cranial right lobe has to be seen just before it, it helps to regognise left from right lung.
The right lung is chosen to work with. So bronchoscope is introduce into the main bronchus as far as possible, that means until third to fifth ventral segmentary bronchi of the caudal lobe, depending on the size of the animal.
At this time, the bronchi are inspected and then infected or flush. When inoculation performed, a 60 ml syringe is used to inject the inoculum through the biopsie canal. When BALs, 4 syringes (240 ml of a 9 p 1000 saline solution + ampicillin + amphotericin) are successively injected and immediately aspirated through the same canal.
In this technique inner tube in the biopsy canal are not used to flush, as described in other experiments. This permit a best fluid return with only few red cells, as no traumatic inner tube. 50 to 90 % of injected saline water with good cells concentration are collected.

The instrument is finally slowly extracted and immediately cleaned with a strict protocol, as mucus and other contaminated particles fix firmly to the plastic when dry. The extern part is first clean with an antiseptic solution, then inner part of the biopsy canal is brushed and washed. A vacuum pump is used to aspirate from distal to proximal part of the canal an antiseptic liquid first and then sterile water, about 1 litre of each liquid is aspirated. This operation is repeated between each BAL.
The BAL aspirated is brought in ice to the lab, then filtered through sterile gaze and centrifuged with 1500 RPM for 10 minutes. If necessary 2 ml lysis buffer can be mixed when red cells contamination. Cells are suspended in 1ml FCS and counted with trypan blue staining. Adding 10 % DMSO they are frozen in aliquots of 5 x 10^6 cells /ml at -80°C . The BAL liquids are also kept aliquoted per 10 ml and stored at -80°C for cytokines dosage.
BALs are performed on days -15, -7, 0, 7,14 for all the animals. Infected group are flushed every week, contact group every 2 weeks until clinical signs and each week after, controls' every 3 weeks.

1-4.2 : Blood sampling.

As possible, once a week (except control group, every 3 weeks) blood samples are taken from all animals under experiment from jugular vein. 100 ml blood mixed with 100 ml alsever solution as anticoagulant are sampled. Because of contamination we give- up using 60 ml syringes to collect. A 16 G needle connected to a sterile perfusion tube which goes into a sterile bottle with alsever is the right solution indeed.
Lymh cells are separated by ficoll (protocol EMVT), aliquoted at 2,5 10 7 cells / ml and storen frozen as describe for BALs.
When necessary 3 .10^6 cells are kept for lymphoproliferation.

1-4.3 : lymph nodes cells.

After slaughtering or natural death, mediastinal and tracheal lymph nodes were extracted to perform lymphoid cells separation. Cells were extracted by scratching nodes with scalpel in RPMI solution and aliquoted at 2,5 10 7 cells / ml and stored frozen.

1-5 : **Miscellaneous.**

The temperature of all the animals are recorded every day or every 2 days depending of the period of the infection. Clinical exams are made every day and coughing and respiratory problems are specially noted. Swabs are made when nasal discharge to check Mycoplasma elimination.
Blood samples for serology diagnostic (CFT and competitive ELISA) for bacteriology department are performed as well.
As far as contamination make it possible, lymphoproliferations are performed as well to measure INF gamma production. This is very helpful in following immune response on each animal.
At last necropsy are performed when animal died or when slaughtered.

2 . **RESULT**

Animal I0 :

This animal displayed all symptoms described for CBPP. Ten days after inoculation (6/10/97) with no BAL before, we noticed hyperthermia and bronchi inflammation in endoscopia, 17 days after the zebu began coughing. Lung lesions were severe 1 month after : narrow bronchi, congestion and exudation. After 3,5 months I0 was still alive with less symptoms but strong macroscopic lesions and irregular temperature. Sacrificed on 9/30/97.
Necropsy : thorax cavity showed no fluid, the right lung were the only affected and posterior part adhered with costalis pleura. A 20 cm sequestrum could be seen in the same area. When cutting external fibrosis capsule, necrotic tissues and pus appeared. Surrounding tissues showed old hepatised lesions with fibrin. Lymph nodes enlarged.

Animal I1 :

Infected with no BAL before on 5/12/97. After 4 days it showed high temperature for almost two weeks (38°C to 40°C). On day 14, nasal discharge, respiratory distress occurred . On 5/31/97 the animal was enable to stand up and was lay down on its right side. It's been decided to sacrifice it the same day.
Necropsy : both side of the thorax were affected.
Right side showed fibrous adhesions between viscerals and parietal pleura. Lung demonstrated a classical feature of CBPP, the interlobular septa distended with fluid separating the hepatised lung lobules and giving the marbled effect. The different degrees of hepatisation gave colours of the lobules from deep red to bluish-grey.
When opening the thoracic cavity left side showed pleural oedema fluid (about 2 litres), no adherence occurred. Lung seemed normal but interlobular septa were slightly distended when cutting.
Lymph nodes were enlarged with fibrous adhesions.

Animal I2 :

Animal infected on 5/12/97 and 6/10/97 as no result in first infection. No BAL performed before inoculation.
Six days after second infection, BAL began very dirty with pus. Bronchial tubes were inflamed. After 1,5 month endoscopic lesions disappeared. Temperature wasn't significant. Sacrificed on 10/1/97.
Necropsy : only right side affected. No fluid when opening but a postero- central sequestrum which adhered costal wall. A cut into a lung demonstrated the marbled effect with necrotic content as well.

Animal I3 :

Animal infected twice as well with no BAL before inoculation (5/13/97 and 6/10/97). No particular clinical signs have been noticed excepted a slight bronchi irritation after both inoculations and 4 days of hyperthermia one week after first inoculation. I3 is still alive.

Animal I4 :

Infected as I3 except that first inoculation was preceded by a BAL. Also 5 days of high temperature after first inoculation. Bronchi inflammation occurred after a week and lasted until slaughtering (10/2/97).
Necropsy : No adherence, no liquid in the thoracic cavity, the right lung had a 5 cm sequestrum in its posterior part. Normal lymph nodes .

Animal I5 :

Infected as I4. High temperature for almost a week, 10 days after each inoculation. BALs with pus and bronchi inflammation with poor clinical signs occurred until slaughtering (10/1/97).
Necropsy : same description as I4.

Animal I6 :

Infected as I5 (5/12/97 and 6/10/97). No clinical sign except slight inflammation after inoculation and some short hyperthermia periods. I6 is still alive.

Animal 855 :

Infection with no BAL before inoculation (5/13/97). Hyperthermia appeared 3 days after inoculation for about a month. On day 13 the animal began to cough, then nasal discharge appeared with severe inflammatory bronchi. This until slaughtering.
Necropsy : on opening the thoracic cavity no adherence and no liquid could be seen. The right lung was the only affected and had a melon size sequestrum in its posterior part. A cut demonstrated a very thick fibrous wall encapsulating the necrotic content which is creamy and caseous. Lymph nodes are thick and enlarged.

Animal 857 :

Same protocol as 855. After 5 days hyperthermia occurred until spontaneous death (6/4/97). On day 14 the animal began respiratory distress with painful respiration, a reluctance to move and a short intermittent cough.
Necropsy : only right side affected with several pleura's adherence. The entire lung showed classical feature of CBPP with interlobular septa distended, the marbled effect and hepatisation.

Animal C1 :

Animal dead on 7/22/97 of an unknown reason.

Animal C2 :

Contact with inoculated animals on 5/15/97. No significant clinical disorder excepted short hyperthermia phases. Sacrificed on 10/2/97.
Necropsy : no adherence and fluid. Characteristic chronic form of CBPP with only left side affected with a melon size sequestrum. The periphery of the lesion demonstrated different degrees of hepatisation. Mediastinal lymph node enlarged.

Animal C3 :

No significant clinical sign. Slaughtering on 10/2/97.
Necropsy : no modification except a slight adherence between pleura and right lung.

Animal C4 and C5 :

No clinical disorder. Animals still alive.

Animal C6 :

No clinical disorder. Slaughtering on 10/3/97 .
Necropsy : no CBPP lesion.

Animal C7 :

First pleurisy sign 2,5 months after contact. In 10 days the animal displayed a acute form of CBPP with respiratory distress. Slaughtering on 8/14/97.
Necropsy : the right side of thoracic cavity showed classical feature of acute CBPP, 1 litre of pleural fluid, strong adherence and almost complete hepatisation. The left side demonstrated just beginning lesions. Lymph nodes enlarged.

Animal C8 :

Bronchial tubes inflammation and mucus exudation 40 days after contact. Slight clinical change. Spontaneous death on 7/14/97.
Necropsy : left thoracic side demonstrated acute lesions with adherences, total lung hepatisation and about 2 litres of thoracic lymph. Right side showed first step of CBPP evolution, few adherence, interlobular septa distended and enlarged lymphatic tract.

Animal C9 :

Same evolution as C8, 40 days after contact 5 days of hyperthermia occurred before severe clinical signs : distress and painful respiration, acute bronchi inflammation. Slaughtered on 7/14/97.
Necropsy : right side affected, adherence, 2/3 of lung hepatised, no thoracic fluid. Lymph nodes enlarged.

Animal C10 :

First respiration difficulties 2 months after contact. Then the zebu showed no aggravation but bronchi exudation until slaughtered on 10/3/97.
Necropsy : Right thoracic side demonstrated a melon size sequestrum in the middle lung, caseum and hepatised tissues when opening. Left side showed an old evolution with fibroblastic reorganisation of the fibrinous exudate. The lung lobules strongly adhered to each other, a thick and caseous fibrinous was deposit over the lung. A cut into the lung showed no particular modification.

Animal C11 :

Animal died on 5/16/97 after sedation. Necropsy demonstrated pericarditis.

Animal C12 :

Coughing and inflammatory bronchi after 1,5 month. Spontaneous death 4 days after first signs (8/3/97).
Necropsy : several litres of yellow thoracic fluid in left thoracic cavity which contained pieces of fibrinous material. Left lung demonstrated acute CBPP lesions, hepatisation and marbled effect.

Animal C13 :

This animal demonstrated an acute form of CBPP 2,5 months after contact : high temperature and severe respiratory distress. 4 days after (8/8/97) sacrifice was decided.
Necropsy : only right side affected. Several litres of pleural oedema fluid with fibrinous material. Posterior lung hepatised, interlobular septa distended, adherence, hydropericarditis. Lymph nodes affected.

Animal C14 :

No evidence of CBPP, excepted 4 days of hyperthermia before spontaneous death (7/26/97).
Necropsy : several litres of fluid with big pieces of fibrinous material in all the thoracic cavity, specially in the left side. Strong adherence with pleura, no significant change of lungs when cutting. Lymph nodes enlarged.

Animal C15 :

Coughing, hyperthermia, severe inflammatory bronchi 3 months after contact. Slaughtering a week after on 8/25/97.
Necropsy : a 25 cm sequestrum was found in right lung, with adherence and no fluid. Nothing abnormal in the left side.

Animal C16 :

1,5 month after contact, coughing and polypnea occurred. No aggravation until accidental death on 9/29/97.
Necropsy : sequestrum on intermediary right lobe, small adherence with pleura . Left side with no modification.

Animal T3 and T4 :

Control animals, no clinical change except slight inflamed bronchi after inoculation of Agar and sterile inoculum.

3 .DISCUSSION

The first problem of the experimentation was the animals. As they have been living in large space for at least 3 years before experimentation, these Zebus could have some difficulties to adapt themselves to be closed as needed. This could explain that some of them couldn't gain weight, were weak and inexplicable deaths.
The second one was contamination. As wet season, a high degree of humidity occurred and samples were easily contaminated. We had real difficulties to manage this, specially with BALs which were almost never sterile even with antibiotics.
Collecting good amount of lymphoid blood cells was also difficult as ficoll separation gives disparate results on zebu.

One can say that infections succeed. Seven animals (77 %) at least from the 9 animals infected had CBPP : 2 acute forms or 22 % (I1,857), and 5 chronic carriers or 55 % (I0,I2,I5,I6,855), I3 and I4 seem to be resistant.
Ten animals (63 %) from 16 contact animals had CBPP : 7 acute forms or 44 % (C7, C8, C9, C12, C13, C14, C15), 3 chronic carriers or 19 % (C2, C10, C16), 4 or 25 % are probably resistant (C3, C4, C5, C6) and 2 or 12 % died of other reason (C1, C11).
Finally at least 68 % of the animals have had CBPP : 36 % of acute form and 32 % of chronic carriers.
Contact animals seemed to develop acute form better than infected animals. In natural infection proportions between acute and chronic are paradoxically clother from infected zebus rather than contacts'.
Caution is needed when calling an animal resistant : 855 and 857 were supposed to be resistant as they did not contract CBPP with infected animals during the first experimentation, in fact they had CBPP immediately after only one inoculation.
Two inoculations were sometime necessary to induce CBPP but second inoculation gave only chronic forms as sensible animals died of acute forms after first inoculation. It's in fact difficult to know if the better rate of animals affected after second inoculation is due to the higher dose of Mycoplasma inoculated or to the booster effect.
BALs didn't seem to influence CBPP development as expected, on contrary better results occurred with no BAL before inoculation in first inoculation.

APPENDIX 12

L.N.I.V. LISBOA
PORTUGAL

PROGRESS REPORT

Dr M.H. FERROHNA

Ministério da Agricultura, do Desenvolvimento Rural e das Pescas

LNIV
Laboratório Nacional de Investigação Veterinária

Departamento de Biologia Celular

CBPP/EU RESEARCH PROJECT

COMPONENT 3: Immunopathology - Immunology

ANNUAL REPORT 1996/97 - Portugal

M. Helena Ferronha, Ana F. Amaro, Ivone Correia

The activities carried out by LNIV during the second year were mainly dedicated to the study of tissues from lung lesions and lymph nodes of bovines, either experimentally infected in Cameroon, in 1996, or naturally infected in Portugal.

The animal ref. 001 was slaughtered in Garoua, in July 96, and the tissue samples were fixed in formaldehyde or frozen at -80°C (as liquid nitrogen was not available at that time) and transported in dry ice to Portugal. The tissues from bovines ref.193, 845, 846, 847 and 917 were fixed in formaldehyde and sent in PBS/sucrose buffer by Dr Herman Unger.

Concerning portuguese animals, either with or without (control) CBPP, tissue samples were immediately frozen in liquid nitrogen after slaughtering, or chemically fixed and processed for embedding in paraffin, to be submitted to immunohistochemistry.

The tissues from african bovines were processed for histopathology, and selected areas were submitted to immunohistochemistry for mycoplasma detection and also for identification of the following immunoglobulins and cell populations, by using the monoclonal antibodies that were kindly provided by Dr. Bensaid:

Immunoglobulins and Cell populations	Monoclonal antibodies
Ig G_1 + Ig G_2	ILA 2
Ig G_2	ILA 73
Ig G_1	ILA 66
Ig M	ILA 50
Ig A	ILA 72
neutrophils	ILA 110
monocytes/macrophages/neutrophils	ILA 24
macrophages/monocytes	ILA 46
B cells *	ILA 65
MHC II	J II
γ/δ T cells	ILA 29
CD8 T cells	ILA 105
CD4 T cells	CD 4 (Serotec)

* recognise also alveolar macrophages in agreement with the information of Dr Bensaid referred in the Annual Report Year 1.

The immunogold tecnique and the streptABComplex/HRP with AEC and the DAB-black substrate chromogens were used as immunolabelling systems.

Some scanner images from photographs are shown in this report with the goal of to exemplify some of the results obtained until now.

A - HISTOPATHOLOGY

Concerning the study of lung lesions from the animals infected in Cameroon, the results of the *post mortem* examination are described in the final report of Dr. H. Unger, presented in the "Intermediary Scientific Report - Workplan nº 2, appendix 7".

A more detailed study was possible in the bovine ref. 001, which lung showed some pleural adhesions, a small sequestrum and a circumscribed area with induration. Microscopically, this lesion consisted mainly of enlarged interlobular septa with oedema, conspicuous fibrin and dilated and frequently ruptured lymphatics with thrombosis. In the pulmonar parenchyma either oedema, fibrin and imflammatory cells within some alveolar lumina, or distended peribronchial and perivascular lymphatic vessels, surrounded by inflammatory cells, were generally observed. These cells also accumulated in some areas marginating the lobules, where demonstrative images of cell degeneration were shown.

Concerning the histopathology of remainder animals, some caution has to be considered, since the tissue samples, might not be representative of the lesion.

However, in lung lesions of animal ref. 846, similar images to those described for the bovine ref. 001 were observed.

In the animals ref. 193, 845, 847 and 917, sequestra with varying forms and sizes were seen. Lesions showing different degrees of septa enlargement, lymphatic ectasis, oedema, fibrin deposition and inflammatory cells were observed.

Contrary to what was shown in lung tissues from portuguese animals with CBPP, numerous eosinophils, stained by the Clayden technique, were visualized in bovines infected in Garoua (fig. 1). On the other hand, the characteristic perivascular organisation foci, frequently observed in the interlobular septa of portuguese infected animals, were never detected in african bovines. These and other findings will be reevaluated after concluding the study of lesions from the animals submitted to the second experimental infection at LANAVET.

B - IMMUNOHISTOCHEMISTRY

• Mycoplasma

The mycoplasma immnunolabelling was tried in lung lesions and lymph nodes from african and portuguese animals by using different methods. The best results were obtained on sections of tissues embedded in paraffin and submitted to the immunogold technique.

In portuguese samples, mycoplasma was detected in the deepest respiratory tract, in the alveolar lumina and within macrophages. Antigens were also detected in lymphatic vessels, including those of distended interlobular septa with thrombi, accumulating in the transition between the pulmonar parenchyma and the interlobular septum. As previously described, the perivascular organising foci were also labelled.

In tissues received from LANAVET, mycoplasma antigens were visualised in lesions of the animal ref. 001, namely in sequestra and within cells present in the alveoli and also in the alveolar parenchyma, near the septum. As mycoplasma detection was not clear in many of the tissues received from Garoua, the data here presented will be completed with the study concerning the second experimental trial .

In the lymph nodes of portuguese bovines, strong reactivity was detected in cells of the subcapsular and medullar sinus, some of them suggesting macrophages. Mycoplasma immunolabelling was also found in the lymphatic follicles, with a heterogeneous pattern of antigen distribution, showing a markedly positive reaction in the periphery of the germinal center (fig. 2).

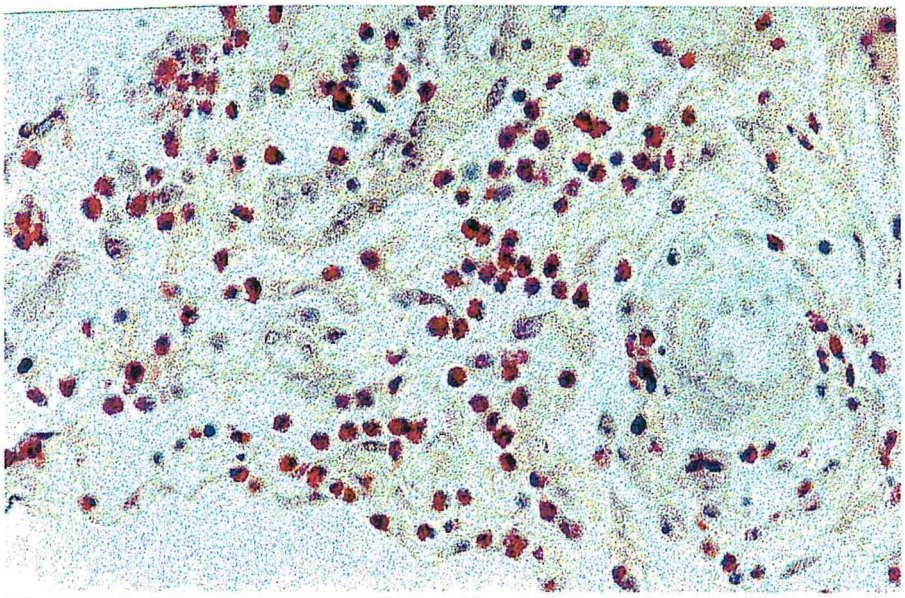


Fig. 1 - Staining of eosinophils by Clayden technique in the lung of animal ref. 001.

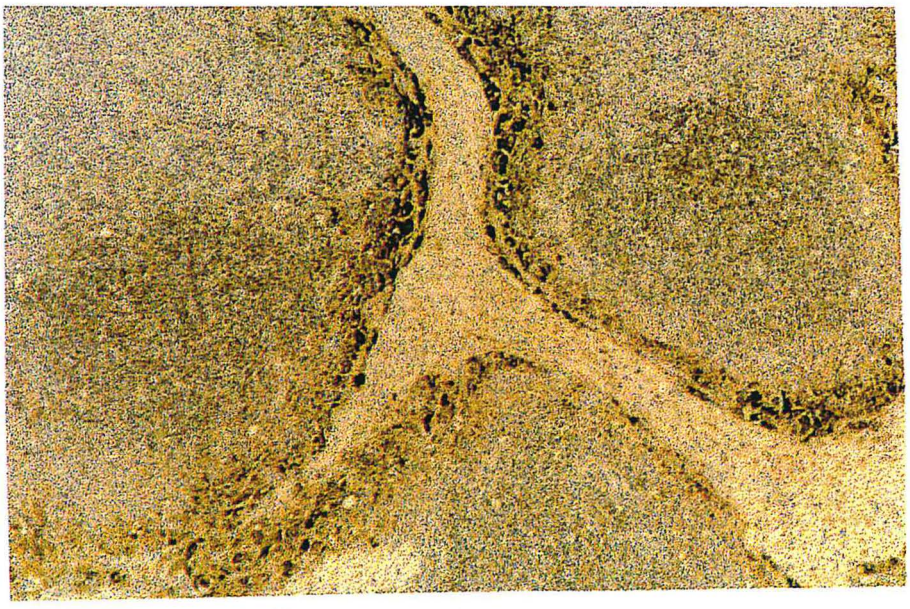


Fig. 2 - Immunolabelling of mycoplasma in the lymph node.

- **Immunoglobulins**

Ig A, Ig M, Ig G_1 and Ig G_2 were detected in lung lesions and their distribution in lymph nodes is under way.

In the lung, of portuguese animals, Ig A positive cells were intensely marked in the lamina propria of some bronchi and bronchioles and few labelled cells were detected in the epithelium. A great number of positive cells were seen surrounding the BALT, the glandular ducts of intrapulmonary bronchi and in the connective tissue beneath the smooth muscle of bronchioles. Immunolabelling was also detected in the pulmonar parenchyma, and in some areas with oedema and accumulation of inflammatory cells near the septum (fig. 3).

In the interlobular septa, Ig A^+ cells were visualised in the foci, generally in the proximity of vessels, but they were never detected in the fibrin or in the necrotic areas. A diffuse labelling of the sero-fibrinous material inside the alveoli was detected, suggesting a free Ig A staining.

In control animals, Ig A was observed in cells present in the connective tissue beneath smooth muscle of bronchioles and in the lumen of bronchioles.

In the animals from Garoua, comparatively to those infected in Portugal, the same partten of Ig A distribution was generally observed, but with less reactivity and more background. Ig A was found inside of some alveoli, in the interlobular septa, within the sequestra and also in few cells scattered in the connective tissue of the capsule.

Concerning Ig M immunolabelling, positive cells were observed in the pulmonar parenchyma, namely in areas with oedema and inflammatory cells. In the septa, few Ig M^+ cells were detected in the periphery of some enlarged lymphatic vessels and near the vessels of the foci (fig. 4). We also found immunolabelling in the lamina propria and in the connective tissue associeted to the bronchi and bronchioles.

In the control, and until now, reactivity was only detected within cells in the connective tissue under brochioles.

In tissue sections from the animals ref. 001, 846 and 917, Ig M positive cells were visualized in the interlobular septa and in association to the bronchioles, like observed in portuguese bovines. Some positivity was found in the alveolar parenchyma but not in sequestra.

In order to detect Ig G in lesions, the monoclonal antibodies ILA 2 (αIg G_1 and αIg G_2), ILA 66 (αIg G_1) and ILA 73 (αIg G_2) were used. No significant differences were seen concerning the two subclasses distribution. The results, although not conclusive, suggest more Ig G_1 than Ig G_2 labelled cells. Further studies are under way to clarify this observation.

A positive diffuse staining was generally detected in the extracellular fluids, usually in areas consisting of oedema and fibrin deposition. Ig G positive cells were seen in the lamina propria of bronchi and bronchioles, surrounding the BALT and the glandular ducts, in the alveoli (with infiltration of inflammatory cells and oedema) and also in the interlobular septa. In the latter, immunolabelling was observed in some cells, either in the foci or in the distended lymphatic vessels, and in the fibrin net (fig. 5).

In control animals, a diffuse staining was mainly detected inside blood vessels and no positive cells were found on the studied sections.

The same pattern of Ig G distribution was shown in the animals infected at LANAVET. Until now, no reactivity was detected within sequestra, although some positive cells were visualised near the capsule.

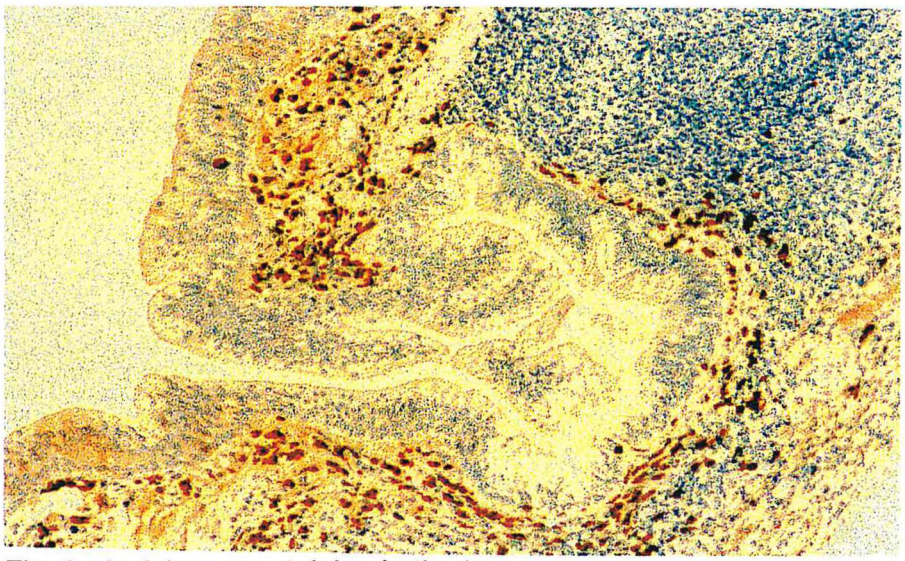


Fig. 3 - Ig A immunostaining in the lung.

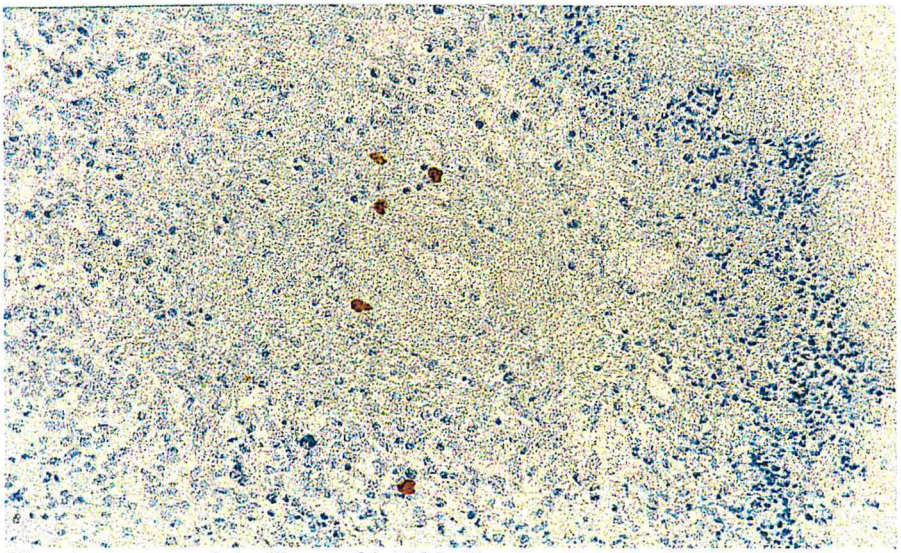


Fig. 4 - Immunolabelling of Ig M in the lung.

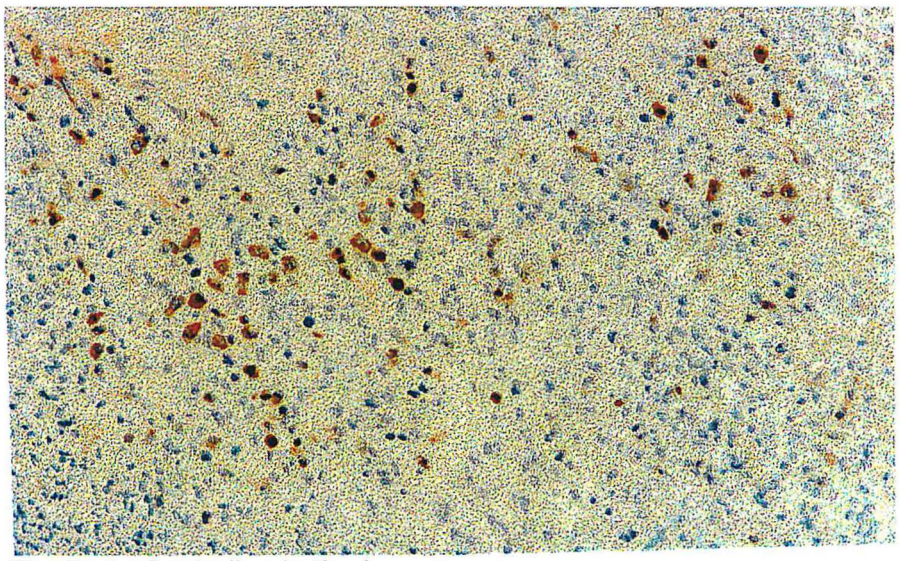


Fig. 5 - Ig G labeling in the lung.

- **Cell populations**

The results concerning the detection of cell populations in tissues, permitted to conclude that specific immunostaining was only observed on cryostat sections of fresh frozen tissues. It is why we did not succeeded in the labelling of tissues from animals ref. 193, 845, 846, 847 and 917. However, in samples from some of these animals, and comparatively to the well preserved tissues from portuguese bovines, a small number of neutrophils were detected on cryostat sections of samples fixed in formaldehyde and frozen in liquid nitrogen. In some samples of bovine 001, it was possible to identify the different cell populations, but it was difficut to establish the spatial relationship between cells and the circumscribed tissues, due to the fact that the tissues were not previously frozen in liquid nitrogen.

Numerous neutrophils spreaded in lung lesions of portuguese animals were observed. Immunopositivity was associated to inflammatory cells generally observed in the alveoli and bronchi, with a lesser extent in the lamina propria and the epithelium. Strong reaction was visualised in the septa periphery, accumulating in necrotic cells. In the enlarged septa, immunolabelling was detected in cells of the foci and in cells scattered in the fibrin, including the thrombi of some lymphatic vessels (fig. 6 and fig. 7).

In control animals few neutrophils were mainly identified in the alveolar walls.

To detect alveolar macrophages, ILA 46 and ILA 24 were used. ILA 46 is specific for monocytes and macrophages but it never worked in the conditions we used. ILA 24 directed to monocytes, neutrophils and macrophages, always showed a lot of background, although permitting to label, with less intensity, generally the same areas stained by ILA 110 (specific for neutrophils).

Concerning lymphocytes, B cells were mainly detected in the BALT associated to some bronchi and bronchioles (fig. 8). This lymphoid tissue was also labelled in control animals, although in a lesser extent.

B cells were detected in the follicles of lymph nodes from the animal ref. 001 (fig. 9).

Immunolabelling of lymphocytes T γ/δ was found in the BALT associated to some bronchioles, in the lamina propria and also below the muscular. Immunoreactivity was also visualised in stuck cells in the fibrin of enlarged interlobular septa and in a lymphatic thrombus. Alone or in aggregates, few T cells γ/δ were seen in the connective tissue and in the pulmonar parenchyma (fig. 10).

In control bovines very few cells immunopositive were visualised in the alveoli.

Infiltration of lymphocytes $CD8^+$ was found in aggregates around blood and lymphatic vessels. Positive cells were detected within the thrombus of an enlarged septa and dispersed in the connective tissue. (fig. 11).

To detect lymphocytes $CD4^+$ in *Bos taurus*, a monoclonal antibody from Serotec was used, giving some background problems. However, these lymphocytes were seen in the BALT, and aggregates of cells were observed in association with some vessels (fig.12).

In preliminar studies effectuated on serial sections, CD4 and CD8 T cells were identified in the similar pulmonar areas, excepting in BALT, where CD8 T cells were not yet deteted.

Concerning the detection of MHC II cells, positive reaction was seen in the BALT (fig. 13), in the pulmonar parenchyma, in a vascular trombus and in some cells trapped in fibrin of the interlobular septa. In the control, none reaction was found until now.

The results described in this report will be added to those concerning the study of lung lesions and lymph nodes from bovines experimentally infected in Cameroon (1997), and, taken together, they will be discussed with the aim of contribute to a better understanding of CBPP immunoparhology.

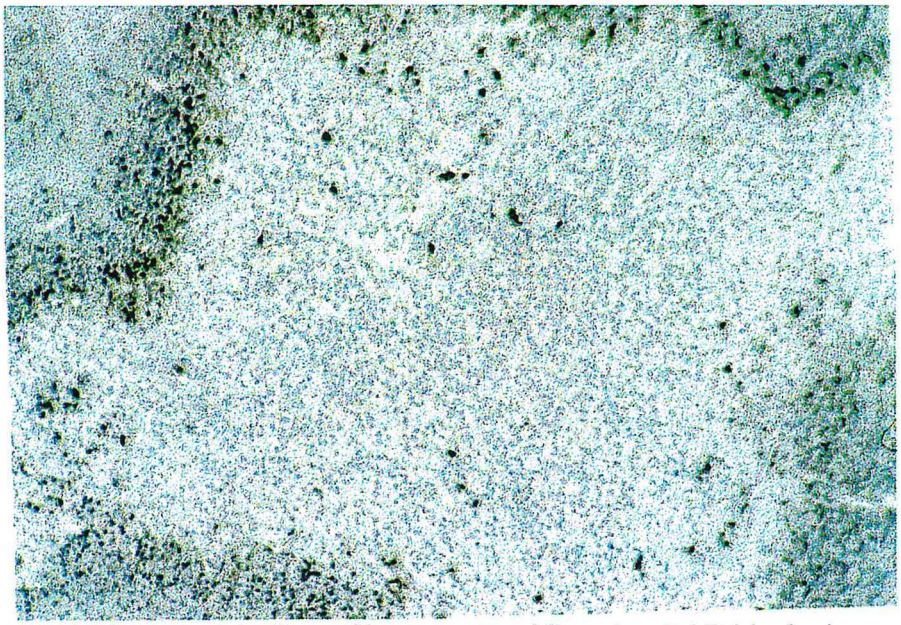


Fig. 6 - Immunolabelling of lung neutrophils using DAB-black chromogen.

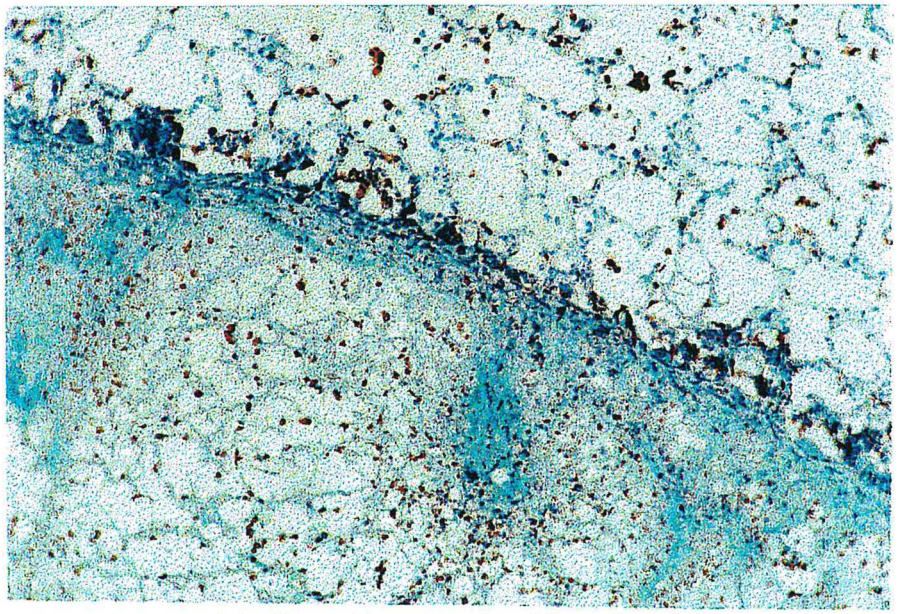


Fig. 7 - Immunolabelling of lung neutrophils using AEC chromogen.

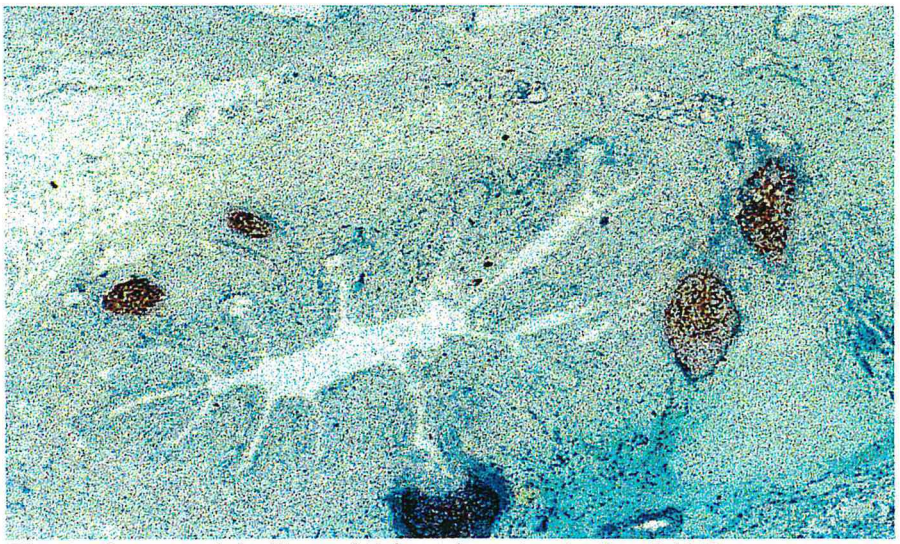


Fig. 8 - Immunolabelling of B lymphocytes in the BALT.

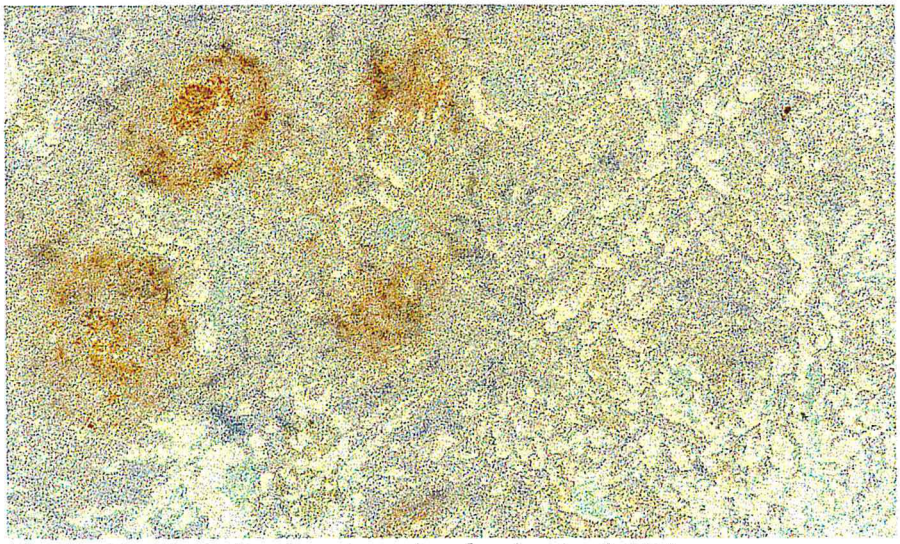


Fig. 9 - B cells in the lymph node of animal ref. 001.

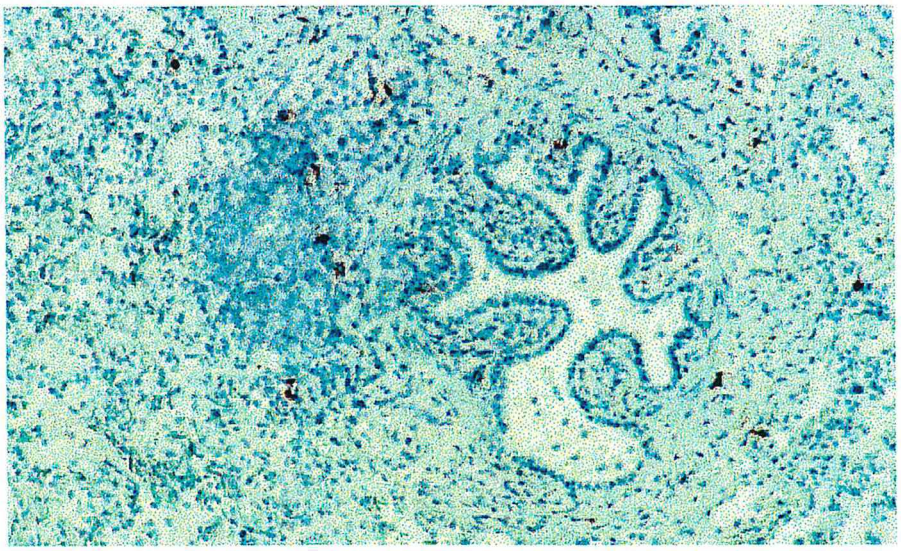


Fig . 10 - γ/δ T cells immunostaining in lung.

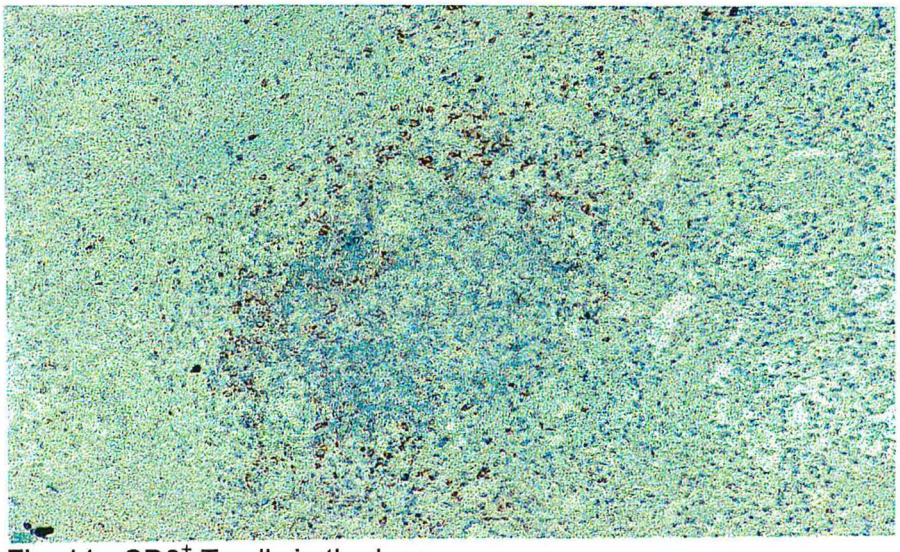


Fig. 11 - $CD8^+$ T cells in the lung.

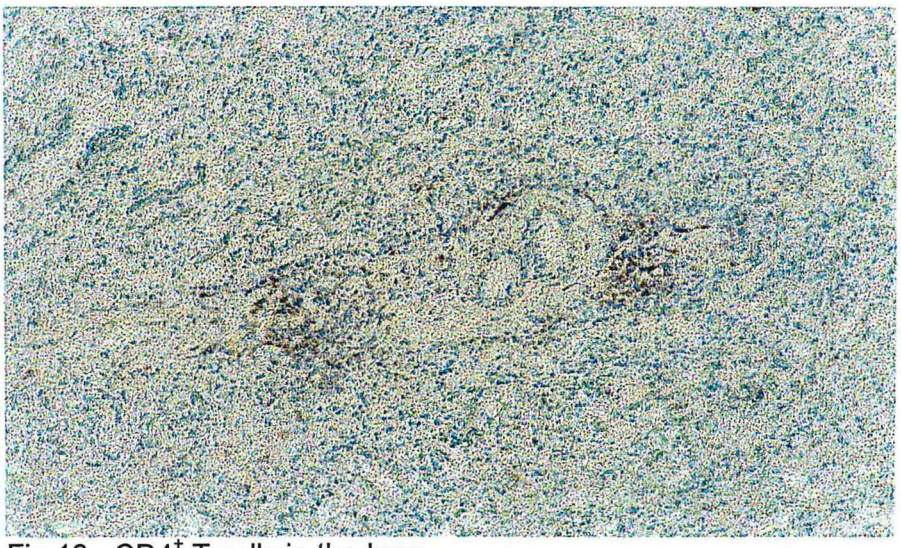


Fig 12 - $CD4^+$ T cells in the lung.

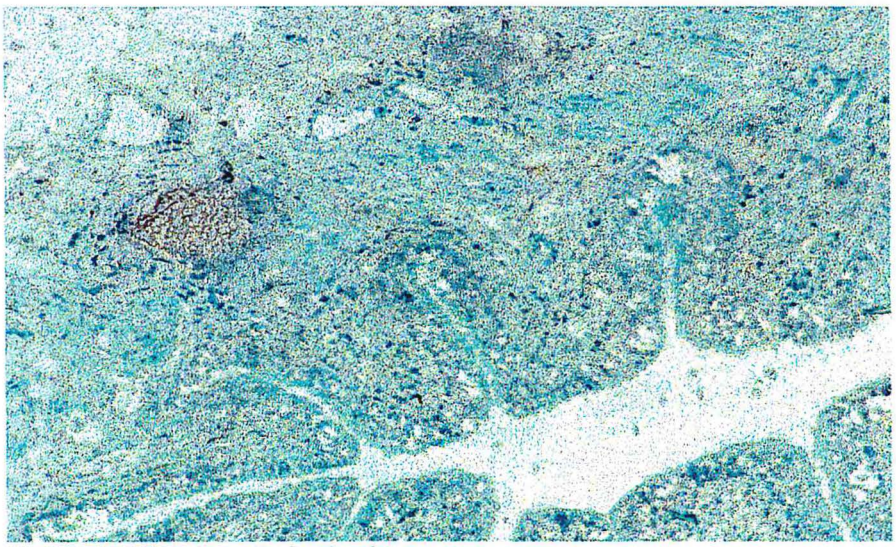


Fig. 13 - MHC II cells in the lung.