SADC SECRETARIAT **EUROPEAN DEVELOPMENT FUND**

Promotion of Regional Integration in the SADC Livestock Sector
(PRINT Livestock Project)

9 ACP SAD 002

Reinforcement of the capacity on Contagious Bovine Pleuropneumonia (CBPP) Diagnostics for SADC Veterinary Laboratories Network

Consolidated report

PRINT Report N°: CBPP--…-2008

Dr. F. Thiaucourt (CIRAD)

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This report is a consolidated and final report pertaining to a consultancy described in the "terms of reference for the reinforcement of the capacity on Contagious Bovine Pleuropneumonia (CBPP) diagnostics for the SADC veterinary laboratories network".

This consultancy was divided in four parts.
1- A preparation phase devoted at contacting all the veterinary laboratories of the SADC
2- An in situ session performed in Lusaka Zambia
3- An in situ session performed in Dar es Salam Tanzania
4- The organization of an inter-laboratory trial devoted to CBPP diagnostic techniques

This consolidated report aims to summarize the main points that arose during this consultancy and that may be helpful to improve CBPP diagnostic capabilities in the SADC region. Very few recommendations were made on the basis that their implementation should have a real beneficiary effect, should be easy put in place and should be easy to monitor.

During the preparation phase of this consultancy it was apparent that, despite a very active collaboration between the veterinary diagnostic laboratories in the field, it was difficult to gather information regarding the veterinary diagnostic laboratories of the SADC.

Recommendation 1

A web site/page should be created to gather all the relevant information on the veterinary diagnostic laboratories of the SADC. Each laboratory should be described briefly but accurately (name of the laboratory, address, phone and fax numbers, Email, director's name, as well as diagnostic tests performed, accreditation, etc). These files should be updated regularly.
See: CD-CBPP diagnostic-SADC/Quality management/Questionnaire_Vetlab-SADC-model/ Lab specifications

Despite actual work in the field by the various veterinary services and diagnostic laboratories, CBPP remains largely underreported to international organizations such as the OIE or the FAO

Recommendation 2

Another site/page should be created to monitor the activities of the various laboratories in the field of CBPP diagnosis (number and type of samples tested, positive results, localization,

typing). Such results should be connected to the epidemiological networks and databases to ease the transmission of information, within the country, at a regional level, and to international organizations.
See: http://www.caribvet.net/

Quality management was stressed by the SADC veterinary diagnostic laboratory network as the ultimate way to improve the reliability of the various laboratories in the SADC, notably for CBPP diagnosis. ISO 17025 was chosen as the ultimate goal. In view of a previous mission performed in 2006 that was devoted to quality management, and in view of the in situ sessions made during this consultancy:

Recommendation 3

The quality management indicator that was used during the 2006 consultancy should be used regularly to monitor the progress made by each laboratory, especially in the field of CBPP diagnosis.
See: CD-CBPP diagnostic-SADC/Quality management/Questionnaire_Vetlab-SADC-model/ Management or Technical or CBPP

The two in situ sessions performed in Lusaka and Dar es Salam have permitted to sensitize the participants to quality management. These sessions have stressed, particularly, the need to identify the "critical points" in each of the techniques that have been demonstrated. This has helped the participants to determine the improvements that may be made in their own laboratories (see the related reports in annexes 2 and 3). However, some important points may be outlined as additional recommendations:

Recommendation 4

Being able to isolate and identify MmmSC is the basis for being recognized as free of CBPP infection as well as to assess the quality of the live vaccines used today.
Hence, every veterinary diagnostic laboratory should be able to perform these tests (as controlled by an inter-laboratory trial)

Recommendation 5

Most diagnostic tests (serology, PCR, titrations…) use micropipettes which should be regularly assessed to ensure the accuracy of the results.
A robust and simple standard operating procedure should be used to assess the accuracy of those micropipettes as well as to monitor the technician's abilities and the accuracy of the ELISA readers. An SOP is included in the CD.
See: CD-CBPP diagnostic-SADC/SOPs/pipette testing

Recommendation 6

PCR is the fastest way to identify CBPP acute outbreaks; however it is prone to contamination problems. Hence great care should be given to the implementation of these tests.
Accordingly veterinary laboratories should switch to Quantitative PCR techniques as soon as these techniques are available and as soon as they acquire the required equipment.

The last part of the consultancy consisted in the organization of an inter-laboratory trial (ILAT) for the various tests used for CBPP diagnosis. Although great care had been given to inform all the SADC veterinary diagnostic laboratories (during the in situ sessions and by E.mail, phone or fax), only 4 of these laboratories took part in the ILAT in parallel with 4

other laboratories in Europe. Most laboratories that took part in this ILAT and that filled the satisfaction enquiry questionnaire, found this ILAT beneficial to their Quality Management.

	Satisfaction enquiry 1	Lab-01	Lab-02	Lab-03	Lab-04	Lab-05	Lab-06	Lab-07	Mean
1	Preliminary information	2	2	2	1	0	3	3	**1,9**
2	Communication with Cirad	-	2	3	2	0	3	3	**2,2**
3	Information delivered on parcel dispatch	2	2	3	1	0	2	3	**1,9**
4	Description of samples	2	2	3	2	0	2	3	**2,0**
5	Quality of samples	2	2	3	2	0	2	3	**2,0**
6	Parcel quality	2	2	3	3	0	3	3	**2,3**
7	Type of transport	1	2	3	3	0	2	3	**2,0**
	Satisfaction enquiry 2								
8	Communication with Cirad on results and reports	-	-	3	3	3	2	3	**2,8**
9	Clarity of final report	-	-	3	3	3	2	3	**2,8**
10	Overall interest of this ILAT for quality management	-	-	3	3	2	3	3	**2,8**

Recommendation 7

Other inter-laboratory trials should be organized in the future for CBPP diagnosis.

To make them easier to manage they may focus on fewer techniques in relation to priorities set by the SADC subcommittee on veterinary diagnostics.

Progress may then be recorded in terms of number of participants and number of participants passing the test(s).

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9 ACP SAD 002

Report of a Mission to the Republic of Zambia Reinforcement of the capacity on Contagious Bovine Pleuropneumonia (CBPP) Diagnostics for SADC Veterinary Laboratories Network

PRINT Report N°: CBPP-ZAM-06-2007

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Gaborone Botswana

Dates: 25th June – 29th June 2007

Reinforcement of the capacity on Contagious Bovine Pleuropneumonia (CBPP) Diagnostics for SADC Veterinary Laboratories Network

PRINT PROJECT
Zambia 25th -29th June 2007

Objective of the workshop
To improve the capacity of each SADC Veterinary Laboratory that is involved in CBPP surveillance and diagnostic in CBPP infected countries, and those in CBPP-free countries which are at risk.

Organizer Institution
CIRAD UPR15 World reference Laboratory for CBPP for FAO, OIE reference Laboratory

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-For PCR session
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Program

Monday
Introduction, medium preparation, culture (liquid, solid media)
Tuesday
Serology, cELISA, pipetting, preparing CFT reagents
Wednesday
Complement fixation Test, Observation of MmmSC cultures, sample preparation for PCR
Thursday
PCR, agarose gel electrophoresis

Friday
General discussion on critical points, harmonized SOPs, round-robin organization

Specific objective of the workshop

Each CBPP diagnostic technique will be implemented in a demonstration with a focus on critical points that affect the quality of the results. These critical points will be discussed by the various participants of the workshop to ensure a list of recommendations to be implemented in the SADC participating veterinary laboratories before interlaboratory testing is organized. Recommendations for the definition of harmonized Standard Operating Procedures will also be issued.

These recommendations and their implementation will be reviewed during the second workshop organized at a later period (September 2007 in Tanzania) by PRINT

Critical points examined

The following critical points were taken for further deliberation and possible recommendations

1. **Media preparation**

a. Work under sterile conditions
b. Make sure to have correct material, e.g. correct Petri dishes for that specific media
c. Validation of media
   -always include negative control
d. Always keep records-worksheet
e. Recycled tubes must be properly cleaned and autoclaved.
f. Recycled tubes that are old and scratched on the outside should be avoided, because it becomes difficult to visualize the culture.

2. **Equipment**

a. Make sure that equipment is properly maintained
b. Use the correct equipment, microscope, etc.
c. Calibrations of pipette

3. C-ELISA

a. The temperature control is crucial for c-Elisa
b. In the kit, the reconstitution of the controls in distilled water is not clearly stated or is not at the space where it is supposed to be.
c. The test should not be read before the controls are validated
d. If one of OD values of any control is aberrant, that OD should be eliminated from the mean.
e. The plate must be covered during incubation- to avoid drying the wells
f. Tips (correct tips for the particular pipette)
g. The values of the controls for each day must be checked and compared to the control values of the previous tests
h. Compare control values of different technicians and kits
i. Always use the correct filter 450nm
j. Keep a back-up lamp for the ELISA reader
k. Always keep records (worksheet)

4. CFT

a. We should all agree at the temperature for de-complementation (inactivation) 56^0C, 58^0C, 60^0C.
b. Always perform complement titration, in order to have a correct working dilution for the test.
c. The incubator must be at correct temperature 37^0C
d. Contaminated/hemolysed samples should not be used as these might give falls results
e. Correct pipetting techniques
f. The test must be validated first by checking the controls

5. PCR

a. Always include controls (pos and neg)
b. Avoid any source of contaminations
c. Preparation of samples, PCR Mix, etc must be done in separate rooms in order to avoid contaminations

d. The pellet must be thoroughly mixed in dist. Water
e. Validate the batch of reagents, each time a new batch is used (see validation protocol given)
f. Tips should be adapted to pipette (correct tips for the particular pipette)
g. Do not store PCR products, they are source of contamination
h. Do not exposure your eyes to direct UV light

6. Quality Control

a. Quality documents must be validated by a second person
b. Traceability of the sample is crucial for quality assurance (keep records)
c. For quality assurance purposes, it is advisable to purchase commercial media
d. Worksheet must be completed at every step of each test.

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9 ACP SAD 002

Report of a Mission to the Republic of Tanzania Reinforcement of the capacity on Contagious Bovine Pleuropneumonia (CBPP) Diagnostics for SADC Veterinary Laboratories Network

PRINT Report N°: CBPP-TANZ-09-2007

Dr. F. Thiaucourt (CIRAD)

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Gaborone Botswana

Dates: 17th sept to 21st sept 2007

Reinforcement of the capacity on contagious bovine pleuropneumonia (CBPP)
Diagnostics for the SADC Veterinary Laboratories Network

PRINT project
Local intervention in SADC
Tanzania, Dar es Salam Sept17th to 21st 2007

Organizer Institution
CIRAD-UPR15   World reference laboratory for CBPP for the FAO, OIE reference laboratory

Experts
Dr F. Thiaucourt (Cirad)

Local organisation
Dr Gabriel Mkilema Shirima (ADRI)

Overall objective of the workshop
To improve the capacity of each SADC veterinary laboratory that is involved in CBPP surveillance and diagnostic, especially those in CBPP-free countries which are “at risk”.

Program

Monday
Introduction, medium preparation, culture (liquid, solid media)
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The two workshops organized in Zambia in June and in Tanzania in September will be followed by an interlaboratory testing that is to take place in the following months. This testing should allow each laboratory to evaluate its own capabilities in terms of CBPP diagnosis and take action for improvement.

THE TRAINING COURSE ON REINFORCEMENT OF THE CAPACITY ON CONTAGIOUS BOVINE PLEUROPNEUMONIA (CBPP) DIAGNOSTICS FOR THE SADC VETERINARY LABORATORIES NETWORK.

REGISTRATION FORM

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ACTIVITIES

Monday Sept 17th

General principles of quality management
External and internal auditing
Ways to write and control SOPs
Training on quality management
Equipment servicing and validation

Recommendations for
ADRI
- Perform training on quality management
- Start by writing some SOPs under QM principles
- Quality policy to be signed and implemented by the direction

Malawi
- Director to sign a QM policy

Botswana
- Verify that the written SOPs are effectively put in action

DRC
- Director to sign a QM policy
- Training is needed
- Servicing equipment

Namibia
- More training on QM
- Equipment servicing

Zambia
- Improve the commitment of direction to implement QM
- Servicing of equipment

Mozambique
- Equipment servicing
- Review the SOPs

Zimbabwe
- Write SOPs for CBPP testing

Angola
- - Director to sign a QM policy

BVI
- ISO 9001
- Start implementing ISO17025 for internal testing

Tuesday 18th

Critical point of serological tests
Selection of suppliers for critical reagents (and how to assess them)
Calibration of micropipettes (theory)
Practical's on micropipette testing according to SOP
- Visualization of errors in pipetting (precision and accuracy)
- Regularity of pipette calibration

Practicals on cELISA
Practicals on Mycoplasma culture (4 different M. species, liquid and solid media)

Recommendations
ADRI
- Perform qualibration
- Improve their knowledge on ELISA reader and ways to send OD values to computer without using EDI: use of "Hyperterminal" using correct BAUD rate (9600)

Malawi
- Select an ELISA reader to work with

Botswana
- Switch from EDI to another software for ELISA data transmission

DRC
- Perform locally some calibration of pipettes

Namibia
- Perform locally some calibration of pipettes
- Implement ways to manage who is using the QM assured equipment

Zambia
- Buy an extra bulb for the ELISA reader
- Calibration of pipettes

Mozambique
- Check the ELISA reader through interlaboratory trial
- Buy a software for transmission of data

Zimbabwe
- Intensify pipette calibration
- Buy a software for transmission of data

Angola
- Testing ELISA reader
- Implement the connection between ELISA reader and computer

BVI
- Intensify technician and pipette calibration
- Buy a software for transmission of data

Results of Pipette check at
ADRI.

A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T
Date:	######			**Pipette multicannaux testée:**				06Y7978		ADRI									
Opérateur:	x,x			**cones / tips:**		Labor-1-100													
Mode opératoire:	Pipette			**colorant : n°**		2007-115													
				Lecteur ELISA		ADRI	x							Acceptability					re
				Filtre:		n°2 : 450								*Global variation*				<5%	W
				Plaque:										*Between mean variation*				<2,5%	W
	07082701-multicanaux12-100µl																		
	1	2	3	4	5	6	7	8	9	10	11	12							
	1,255	1,268	1,252	1,266	1,276	1,243	1,118	1,275	1,278	1,271	1,265	1,267							
	1,258	1,256	1,256	1,257	1,278	1,235	1,107	1,271	1,271	1,272	1,283	1,268							
	1,254	1,252	1,261	1,253	1,272	1,237	1,162	1,269	1,272	1,275	1,273	1,262			CV				
	1,244	1,274	1,268	1,272	1,262	1,247	1,085	1,278	1,276	1,262	1,277	1,274			4,202				
	1,241	1,268	1,271	1,265	1,27	1,241	1,124	1,277	1,289	1,277	1,268	1,268							
	1,566	1,256	1,265	1,246	1,266	1,255	1,125	1,274	1,284	1,277	1,269	1,26							
	1,253	1,256	1,26	1,266	1,274	1,246	1,125	1,278	1,267	1,273	1,273	1,268							
	1,263	1,268	1,265	1,261	1,27	1,238	1,116	1,266	1,26	1,264	1,276	1,262							
																	% variation entre		
Max	1,566	1,274	1,271	1,272	1,278	1,255	1,162	1,278	1,289	1,277	1,283	1,274		1,566			*moyennes*		
Min	1,241	1,252	1,252	1,246	1,262	1,235	1,085	1,266	1,26	1,262	1,265	1,26		1,085			*de colonnes*		
moynne	1,292	1,262	1,262	1,261	1,271	1,243	1,120	1,274	1,275	1,271	1,273	1,266		1,256	1,292	1,120	13,657		
ecartype	0,1110	0,0081	0,0063	0,0084	0,0052	0,0065	0,0215	0,0044	0,0093	0,0056	0,0057	0,0045		0,0528					
(Max-Min)/moy	0,252	0,017	0,015	0,021	0,013	0,016	0,069	0,009	0,023	0,012	0,014	0,011		38,302					
														Pourcentage de variation total					

Multicannaux 12 - 50-300µl

Max, Min, Moy sur chaque colonne

This first trial of multichannel 06Y7978 yielded unsatisfactory results:
- There is one aberrant value in column 1 6th well (OD 1.566)
- All values from column 7 are bellow the values of other columns.

These non-conformancies could be attributed to the multichannel pipette itself or the incorrect fitting of the tips.

A second check was performed two days later with the same pipette

A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S
Date:	70920			**Pipette multicannaux testée:**				06Y7978		ADRI								
Opérateur:	F. Thiaucourt			**cones / tips:**		Labor-1-100												
Mode opératoire:				**colorant : n°**		2007-115												
				Lecteur ELISA		ADRI	x							Acceptability				res
				Filtre:		n°2 : 450								Global variation				<5% Ok
				Plaque:										Between mean variation				<2,5% Ok

07082701-multicanaux12-100µl

	1	2	3	4	5	6	7	8	9	10	11	12				
	1,131	1,136	1,141	1,145	1,164	1,149	1,147	1,159	1,147	1,136	1,153	1,152				
	1,133	1,125	1,134	1,136	1,138	1,135	1,141	1,136	1,14	1,132	1,123	1,128				
	1,125	1,135	1,137	1,135	1,135	1,136	1,139	1,133	1,125	1,121	1,123	1,127		CV		
	1,136	1,127	1,135	1,133	1,141	1,137	1,132	1,135	1,139	1,136	1,137	1,148		0,677		
	1,138	1,133	1,137	1,131	1,134	1,135	1,133	1,134	1,135	1,136	1,139	1,147				
	1,131	1,127	1,13	1,133	1,137	1,14	1,132	1,133	1,133	1,136	1,14	1,142				
	1,137	1,125	1,13	1,127	1,128	1,134	1,125	1,128	1,12	1,133	1,131	1,13				
	1,134	1,131	1,131	1,134	1,138	1,133	1,129	1,141	1,144	1,142	1,14	1,152				
																% variation entre
Max	1,138	1,136	1,141	1,145	1,164	1,149	1,147	1,159	1,147	1,142	1,153	1,152	1,164			moyennes
Min	1,125	1,125	1,13	1,127	1,128	1,133	1,125	1,128	1,12	1,121	1,123	1,127	1,12			de colonnes
moynne	1,133	1,130	1,134	1,134	1,139	1,137	1,135	1,137	1,135	1,134	1,136	1,141	1,136	1,141	1,130	0,958
ecartype	0,0042	0,0045	0,0039	0,0051	0,0107	0,0052	0,0071	0,0095	0,0092	0,0060	0,0100	0,0108	0,0077			
(Max-Min)/moy	0,011	0,010	0,010	0,016	0,032	0,014	0,019	0,027	0,024	0,019	0,026	0,022	3,875			
													Pourcentage de variation total			

Multicannaux 12 - 50-300µl

DO: 1,1 – 1,3; lignes: 1–8

col 1, col 2, col 3, col 4, col 5, col 6, col 7, col 8, col 9, col 10, col 11, col 12

Max, Min, Moy sur chaque colonne

DO: 1,1 – 1,3; Colonnes: 1–12

Results were then passing the threshold values.

These results show that:

1) Pipette 06Y7978 passes this quality check
2) The previous individual aberrant value may have occurred due to incorrect pipetting
3) The previous aberrant values for channel N°7 was due to an incorrect fitting of the tip

As a consequence, the SOP for the multichannel pipette 06Y7978 should mention that the tips should be well adjusted before starting pipetting.

cELISA demonstration

Results
The initial values given by EDI software were outside limits (MAb OD too low)
Analysis of the problem showed that the EDI software was unable to set the correct filter of the ELISA reader.
After entering the correct wavelength directly on the ELISA reader, cELISA values were all returning to acceptable values.

As a conclusion
1) The cELISA kit proved very robust as it gave correct values although it was kept at the custom without cooling for more than 7 days.
2) Laboratories performing ELISAs should ensure that they are using correct settings, hence the importance of operating manuals for each equipement and software.

Wednesday 19th

PCR sample preparation
Comparison of advantages and drawbacks of PCR and isolation
Q principles of Plan Do Check and Act
Continuous training for technicians is needed and evaluation of training is also needed (qualification of trainer and trainees)

Practicals on PCR for CBPP (it worked!)

ADRI
- Training on PCR is needed as very good equipment and facilities exists

Malawi
- Find facilities and agreement to perform PCR

Botswana
- Continuous training on PCR needed

DRC
- Purchase the necessary reagents for CBPP PCR

Namibia
- Training on QM of PCR technique

Zambia
- Reinforcement of the links with other institutions performing PCR

Mozambique
- PCR technique has to be established

Zimbabwe
- Write SOPs for CBPP PCR

Angola
- PCR technique has to be established

BVI
- Use of PCR for checking cell culture contaminations
- Use of PCR for checking T1 vaccine identity

PCR exercise pointed out the need to manipulate with the correct safety precautions Etidium Bromide (use of gloves, SOPs to eliminate gels…)

In spite of incorrect storage at custom for more than 7days, the PCR results were correct. This shows the robustness of the technique and its usefulness in case of emergency situations.

The main drawback of PCR is the risk of contaminations which was discussed at length during the exercise

Thursday 20th

Agarose gel electrophoresis (Various technical points)
Safety precautions for EtBr manipulations
Review of the advantages and drawbacks of PCR

CFT, review of the various critical points for the CFT
(temperature for decomplementation, Sheep red blood cells, complement activity…)
Practicals on complement activity testing
Re-testing pipettes from ADRI using hyperterminal as a way to transmit data

Controls of Mycoplasma cultures (solid: aspect of colonies, liquid: pH indication)

ADRI
- Improve stereo microscope
- Improve the SOPs for medium preparation and control of quality of medium
- Seed larger plates to be able to perform isolation
- Modify SOP for Complement titration
- Records of laboratory work have to be improved

Malawi
- Procure reagents for isolation of mycoplasmas
- Training for CFT

Botswana
- Obtain ready made medium and compare with locally made medium
- Monitor better the critical points of the CFT

DRC
- Training on CFT
- Purchase of medium to implement isolation

Namibia
- Reagents needed for isolation, training
- setting SOPs for CFT

Zambia
- Stereomicroscope needed

Mozambique
- Buy medium for isolation
- Need of reagents

Zimbabwe
- Training on culture and identification (and CFT)
- Procurement of reagents (CFT and isolation)
- Stereomicroscope needed
- Control cultures needed

Angola
- Reagent procurement (CFT and isolation)

BVI
- Purchase of reagents for testing vaccines

The complement titration performed on Thursday 20th failed to give proper values as no well showed complete haemolysis.

A first hypothesis was that the haemolytic system had not been prepared correctly or that the agitation was not sufficient.

A second trial failed to give proper results again.

The most likely conclusion was that the complement activity had been lost during the improper storage of the kit at the custom.

Recommendations could be as follow:
1) Ensure that procedures to retrieve parcels at the custom are fast enough
2) Keep Guinea pigs as alternative source of complement

Interlaboratory trials

- Emails sent to Heads of labs on
- Official answers still awaited for many labs
- Official letters to be sent by fax or DHL

Interlaboratory trial to be organized between October November 2007
Report of interlaboratory trial to be sent before the end of December 2007

Need to repeat interlaboratory trials within SADC vet lab network

SADC SECRETARIAT **EUROPEAN DEVELOPMENT FUND**

Promotion of Regional Integration in the SADC Livestock Sector (PRINT Livestock Project)

9 ACP SAD 002

Reinforcement of the capacity on Contagious Bovine Pleuropneumonia (CBPP) Diagnostics for SADC Veterinary Laboratories Network

Interlaboratory Trial (CBPP-ILAT-2007)

PRINT Report N°: CBPP--…

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Dates: 15th oct to 21st dec 2007

1. INTRODUCTION

Under the framework of the PRINT project and the SADC Network of Veterinary Laboratories, CIRAD-UPR15, world reference laboratory for CBPP for the FAO and associated reference laboratory (with AFSSA Lyon France) for the OIE, has organized an interlaboratory trial (ILAT) on contagious bovine pleuropneumonia (CBPP).
This ILAT was organized within the framework of ISO17025, chosen as a final goal by the SADC Network of Veterinary Laboratories. This norm specifies that assuring the quality of a test can be achieved by the participation to interlaboratory comparisons and proficiency testing. This ring trial was organized in compliance with the French norm NF U47-400 "Guidelines for the organization of interlaboratory trials in animal immunoserology habilitation" [1] as well as the guides Edited by the OIE [2]. Whenever possible the samples designed for this trial were chosen to assess Sensitivity, Specificity and Repeatability.

2. MATERIAL AND METHODS

Six different tests were proposed for this ILAT. The first test was designed for assessing the accuracy of pipetting with multichannels pipettes. This was considered as a preliminary test before assessing the quality of ELISA performance. The second test was meant to assess the ability to cultivate and identify *Mycoplasma mycoides* subsp. *mycoides* SC (MmmSC), the agent of CBPP. Isolation and identification of MmmSC are considered of crucial importance for confirmation of CBPP outbreaks in many countries as well as for the evaluation of vaccine quality. The third test consisted in PCR identification of MmmSC. In that case what was evaluated was the ability of the labs to perform a test allowing the identification of CBPP outbreaks in an emergency situation. Finally the last three tests were considering the serological tests that can be applied for the confirmation of CBPP outbreaks, for estimating prevalence or for establishing freedom of infection i.e.: Complement Fixation Test (CFT), cELISA and Immunoblotting (IBT).

In total 8 laboratories participated to this ILAT, including CIRAD as the organizing laboratory. These laboratories were located in the SADC region in Africa (N=4) as well as in Europe (N=4). For the purpose of this report, they were numbered randomly from 01 to 07, only CIRAD's results being made openly public.

2.1 Organization of the ILAT

The principle of this ILAT was decided during the discussions with the SADC Network of Veterinary Laboratories and the PRINT project in early 2006. A preliminary assessment of quality assurance in the various veterinary laboratories of the SADC was performed by sending a questionnaire to all laboratories and later-on by implementing a visit in four countries (Botswana, Mozambique, Zambia, Zimbabwe). Sensitization and training to quality management in CBPP diagnosis was performed in 2007 through two in-situ workshops, through the PRINT project, in Zambia and Tanzania respectively. These workshops were attended by 20 participants from various SADC veterinary laboratories and consisted of an analysis of critical points for CBPP diagnosis techniques.
Following the last workshop, an official announcement of the ILAT was sent to all SADC veterinary laboratories on 05/09/2007. This announcement was sent by E.mail and then by fax or by phone for those laboratories that failed to send an acknowledgement of receipt.

Parcels containing the various samples were sent by courier on 23/10/2007. The courier allowed the tracking of the parcel. Laboratories were informed of the parcel dispatch and content by E.mail.
A preliminary satisfaction enquiry was sent on 08/11/2007 to evaluate this first part of the ILAT and a second satisfaction enquiry will be sent by the end of December once all laboratories have received this report.

2.2 Pipetting accuracy testing

This test had to be performed with a standard operating procedure (SOP) describing how to check regularly the micropipettes. This SOP does not replace the official pipette testing that is performed within an accreditation procedure. It has been designed to monitor regularly the quality of pipetting and to be able to spot any deviation to acceptable results. It can be used to monitor the pipettes, the tips or the ELISA reader. It has been designed to validate the pipetting around 10 and 100 µl that are the volumes used when performing cELISA for contagious bovine pleuropneumonia.
The assay consists in measuring optical densities of a yellow dye which was supplied by CIRAD as a concentrated solution. Repetitions of measurements are made to evaluate the accuracy and precision of this measurement by transferring raw results into a spreadsheet. In the case of a multichannel pipette, 96 measurements are analysed and consist of calculating, for the whole plate or for each row: the minimum OD, the maximum OD, the mean OD, the coefficient of variation (std/mean) and the maximum acceptable deviation (MAD) (=(Max-Min)/mean).

2.3 Cultivation and identification of MmmSC

Four freeze dried strains were sent to each participating laboratory. They consisted of four mycoplasma strains: MmmSC (strain PO67), *M. capricolum* subsp. *capricolum* (strain 7714), *M. putrefaciens* (strain Tours2) and *M. mycoides* subsp *mycoides* LC (MmmLC, strain 95010).
The aim was to identify if any of the vials was containing an MmmSC strain and to evaluate the titre in this vial.
As a preliminary trial, CIRAD had quantified the content of the MmmSC vials and assessed the stability of the product after 44 hours at various temperatures (°4°C, 20°C, 37°C). Titration at CIRAD was performed on solid medium (modified Gourlay with sodium pyruvate) by seeding 20µl of tenfold dilutions of the reconstituted vial, according to OIE manual.

2.4 Detection of MmmSC by PCR

Four DNA samples were sent to each laboratory. They consisted in two positive DNA samples from vaccine strain T1/44. One of the positive DNA sample was highly concentrated while the other was diluted as to spot possible lack of sensitivity. Two other DNA samples consisted of MmmLC DNA from strain 95010. MmmLC was chosen as this subspecies is the most likely to lead to false positive results by PCR.

2.5 Detection of MmmSC antibodies in serum samples (by CFT, cELISA or IBT)

The samples consisted in three different groups of samples. The first consisted in 6 sera of French origin which is free of CBPP as to have 6 negative controls. The second consisted in 6

known positive serum samples originating from a CBPP experimental reproduction that took place in Cameroon (LANAVET, Garoua) in 1998. Animals taking part in this experiment had been kept in observation for more than four weeks and did not show any sign of any disease prior to the experiment. They were slaughtered at the end of the experimental reproduction of CBPP. These sera were kept at CIRAD in a freeze dried form to ensure their stability upon storage. Some of these sera were included in duplicate to evaluate the repeatability of the testing. Furthermore these sera were added with a preservative to ensure their good conservation upon transport. Preliminary trials were performed at CIRAD to ensure that this preservative was able to inhibit the growth of contaminants such as *E. coli* and that it allowed a good conservation of the sera even after 7 days at 37°C. In total 10 positive sera were included in this group.
Finally the third group of sera consisted in 8 dilutions of a known highly positive serum sample (diluted in negative bovine serum).
All sera (N=24) were distributed in 1.5 ml screw-capped tubes and identified by a unique random number to ensure anonymity of samples.

For CFT, results were expressed by the various laboratories, according to standard rule, as the last dilution giving an inhibition of haemolysis and expressing this inhibition from 0 to 4+. These results were transformed into an arithmetic scale to ensure statistical analysis (Annex1). This transformation ensures that the variance of the results is not dependant of the titre and then allows valid statistical comparisons. The cut-off for this test is 4+ at 1/10 or, expressed in the arithmetic scale: >= 4.

For cELISA results were expressed in percentage of competition, the cut-off for this test is 50% and titres ranging from 40 to 50 are considered as dubious.

For immunoblotting results are only qualitative and a serum is found positive only if it shows five specific bands of 110, 98, 95, 62/60, 48 kDa size.

3. RESULTS

3.1 Management of the ILAT

Out of the 7 laboratories some of them chose not to implement all the proposed techniques. Table 1 summarizes the number of laboratories that expressed the wish to perform the tests and the actual number of answers received after the final deadline for result submission.
Communication problems were encountered with one of the laboratories. The fault is partially due to CIRAD as communication relied solely on E.mails for sending documents. One of the laboratories had an interruption of telephone lines which did not allow a good reception of instructions. In total, more than 150 E.mail messages were exchanged at the occasion of this ILAT.
Besides, one of the laboratories apparently did not receive the freeze dried samples. Although all precautions were taken when packing the samples an error is always possible. We should have included a precise packing list that should have been used as an acknowledgement of receipt to be sent back by fax on the day of reception.

Test	Description	Expected Number	Received on time	Percentage
A	Pipetting accuracy	4	3	75
B	MmmSC cultivation & ID	7	4	57
C	PCR	6	5	83
D	CFT	6	5	83
E	cELISA	4	3	75
F	IBT	3	2	67

Table 1: Summary of expected number of results per type of test and actual number received

The satisfaction of the “clients” as per the organization of this ILAT was evaluated before final results were issued. Evaluations varied greatly from one laboratory to another. The worse score was obtained with the laboratory that had E.mail reception problems (Lab-05), which is quite understandable. For another one (Lab-01) transport was considered as a problem. This was due to the carrier that failed to deliver the parcel on time (the parcel arrived at the end of the week instead of a Monday or Tuesday as planned). Without these exceptions, satisfaction was high (Table 2)

Satisfaction enquiry 1	Lab-01	Lab-02	Lab-03	Lab-04	Lab-05	Lab-06	Lab-07	**Mean**
Preliminary information	2	2	2	1	0	3	3	**1,9**
Communication with Cirad	-	2	3	2	0	3	3	**2,2**
Information delivered on parcel dispatch	2	2	3	1	0	2	3	**1,9**
Description of samples	2	2	3	2	0	2	3	**2,0**
Quality of samples	2	2	3	2	0	2	3	**2,0**
Parcel quality	2	2	3	3	0	3	3	**2,3**
Type of transport	1	2	3	3	0	2	3	**2,0**

Table 2: Satisfaction enquiry results for the organization of this ILAT. Scores ranged from 0 to 3 for “Bad”, “Acceptable”, “Good” and “Very good”.

3.2 Pipetting accuracy

The three laboratories that performed this test had quite variable results as shown by the graphs in Figure 1 for the multichannel pipettes.

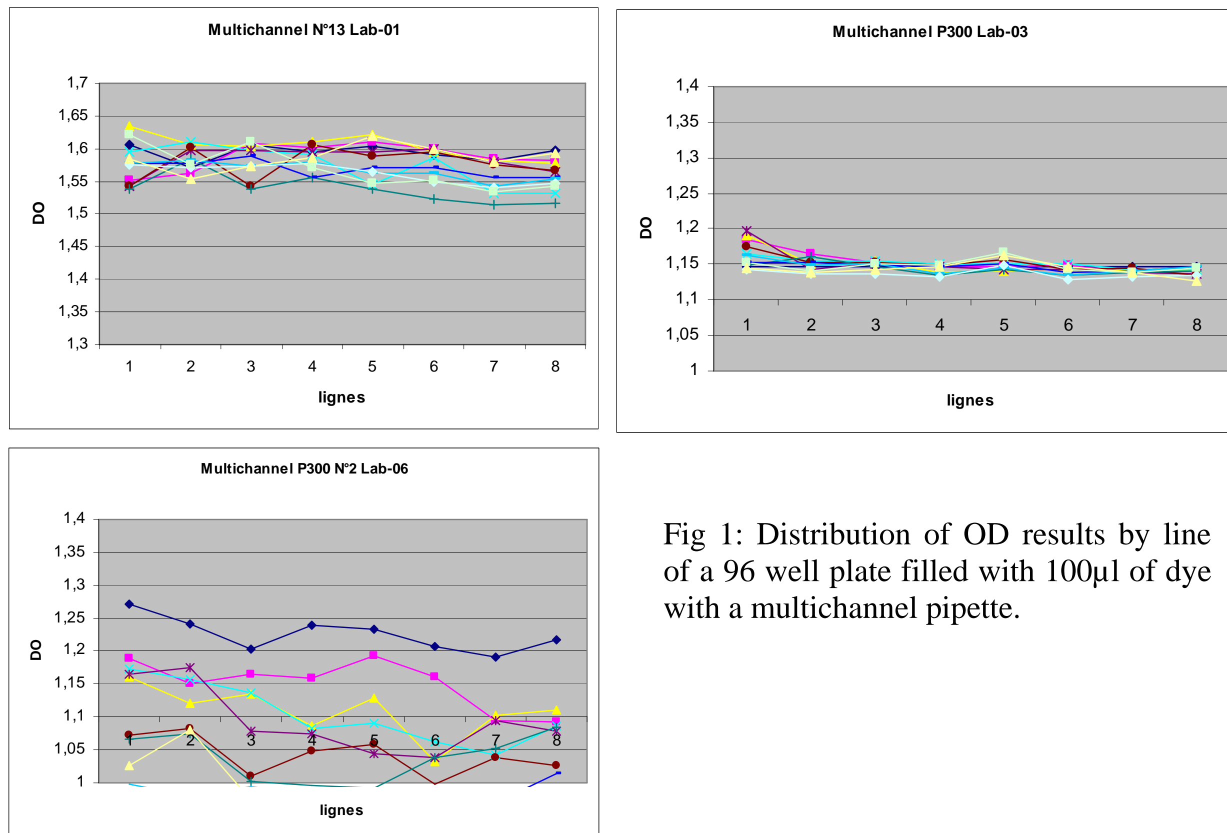


Fig 1: Distribution of OD results by line of a 96 well plate filled with 100µl of dye with a multichannel pipette.

Lab-03 failed for the test of Maximum Acceptable Deviation simply because of the first two wells of the first line that had ODs significantly above the mean. In that case this variability could come from a problem with the initial "wetting" of the tips.

Lab-01 had results which were much more variable and Lab-06 even more variable. This tends to indicate that the situation could be improved by carefully checking the fitness of the tips with the multichannel or checking if various technicians find similar results or even check the ELISA reader (with a reference plate).

		Lab-01		Lab-03		Lab-06	
"Maximum Acceptable Deviation"	<5%	7,6	fail	6,1	fail	37,1	fail
Mean Maximum acceptable Deviation	<2,5%	4,1	fail	1,4	PASS	29,1	fail
CV	<1,5%	1,6	fail	1,0	PASS	9,2	fail

Table 3: Final results for pipetting accuracy check.

Inaccuracy of pipetting is the primary cause of variation for serology results and corrective actions should certainly be implemented in the laboratories that did not pass the test (01 and 06).

3.3 2.3 Cultivation and identification of MmmSC

Tests performed at CIRAD showed that the initial titre in the freeze dried product was around 6.6 log CFUs. Storing vials at various temperatures for 44 hours did not have a significant effect (+4°C or +20°C) on titre except for the storage at 37°C which resulted in a loss of about 1 log. To take this possible drop of titre during transport into consideration, we considered the final result to be correct if >= 5logs.
Only two laboratories had satisfactory results (04 and 07). One identified correctly MmmSC (Lab-02) but did not perform the titration. Most of the others did not perform the test and a single one performed the test but failed to identify MmmSC (Lab-05).

Table 4: MmmSC cultivation and identification results

	Identification	Titre	Specificity	Sensitivity
CIRAD	MmmSC	6,6		
Lab-01	Not done	Not done	Fail	Fail
Lab-02	OK	Not done	**PASS**	Fail
Lab-03	Not done	Not done	Fail	Fail
Lab-04	OK	5,00	**PASS**	**PASS**
Lab-05	Wrong		Fail	
Lab-06	Not done	Not done	Fail	Fail
Lab-07	OK	6,40	**PASS**	**PASS**

Analysis
The lack of results for many laboratories for this test is quite disappointing as isolation of MmmSC remains a crucial parameter for a better knowledge on CBPP distribution and impact measurement. When culture methods are correctly applied, culture takes only a few days and identification can follow with PCR or real-time PCR.
Isolation can be used with field samples and confirmation of CBPP in previously non infected zones. It can be used in infected zones to monitor the possible occurrence of antibioresistance. It can be used in vaccinated areas to analyze the possible cases of post-vaccinal reactions and allow their confirmation or, at the contrary, show that animals were dying of a pre-existing pathogenic MmmSC strain. Finally MmmSC cultivation is absolutely necessary for the evaluation of CBPP vaccines that are used in the field.

3.4 Detection of MmmSC by PCR

Out of 6 laboratories that intended to perform PCR for the detection of MmmSC, 1 failed to perform the test. Four laboratories that sent PCR results identified correctly the two vials that contained MmmSC DNA at two concentrations. Furthermore two of them correctly identified MmmLC DNA in the other two vials. One of the laboratories failed to identify the sample that contained the lowest concentration of MmmSC DNA. This reveals a lack of sensitivity in this laboratory which should be corrected.

Table 5: Identification of MmmSC DNA by PCR

	MmmSC ++	MmmSC +	ID of other DNA	Specificity	Sensitivity
Lab-01	Not done	Not done	Not done	Fail	Fail
Lab-02	OK	OK	OK	**PASS**	**PASS**
Lab-03	OK	OK	Not done	**PASS**	**PASS**
Lab-04	OK	OK	Not done	**PASS**	**PASS**
Lab-05	OK	Wrong	Not done	**PASS**	Fail
Lab-07	OK	OK	OK	**PASS**	**PASS**

Analysis

PCR has become in the recent years a universal technique with multiple applications. CBPP is no exception and more than 5 different PCR protocols have been proposed for a specific identification of MmmSC strains. In addition, one PCR protocol has been proposed for the specific identification of T1 vaccine strain as a help for vaccine identity check and for the identification of strains involved in post vaccinal reactions.

Efforts should be made in Africa to help laboratories that wish to implement this technique. This would be of paramount importance for countries that are free of CBPP but at high risk of introduction. However it is well known that classical PCR poses a lot of problems in diagnostic laboratories because of the risk of contamination. Efforts should be made to promote the use of real-time PCR that may present a lower risk of contamination.

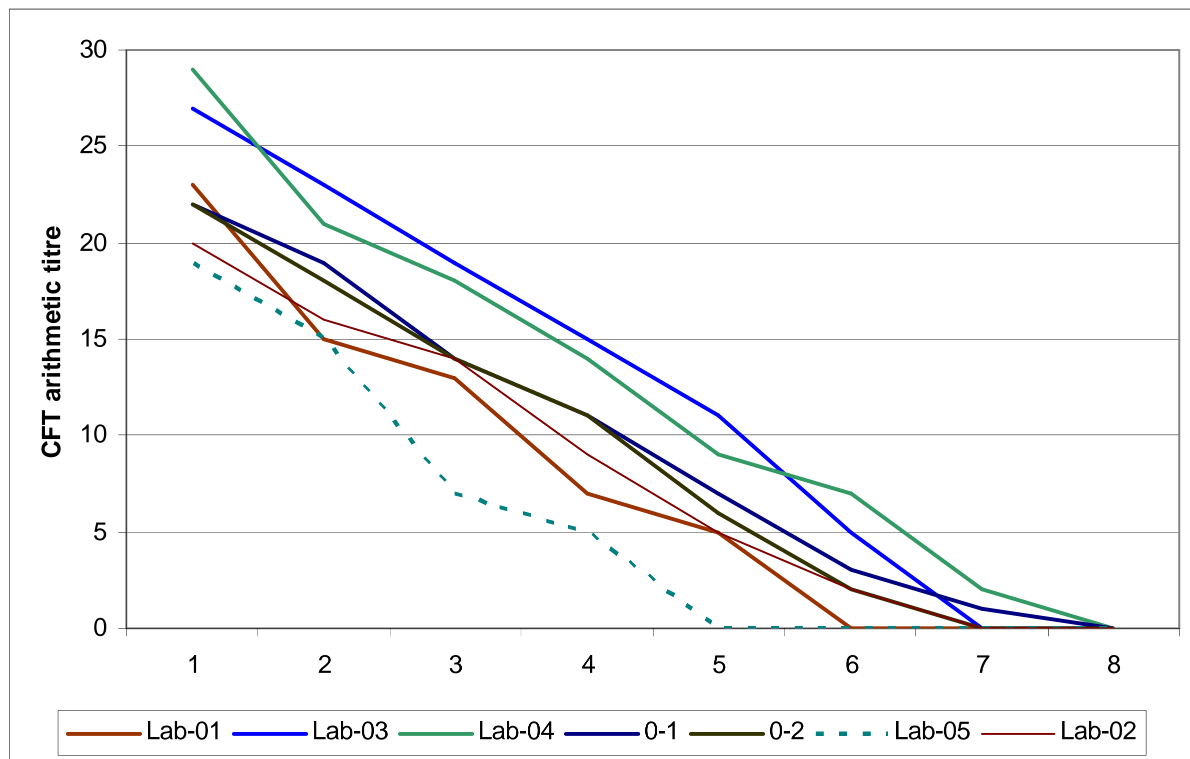
3.5.1 Serology, complement fixation test (CFT)

Six laboratories had intended to participate to the CFT comparisons. Out of them, one failed to send appropriate results on time with all sera tested and titres expressed.

The two curves obtained at CIRAD (Figure 2: curves 0-1 and 0-2) for the dilutions of the highly positive serum with or without preservative were strictly similar. This shows that this preservative had no detrimental effect on CFT results. Earlier results had also shown that these titres were stable for more than one week at 37°C.

For the highly positive serum given in dilutions, analysis of the results consisted in checking the regularity of the slope as well as the titres given for each dilution. The curves shown in Figure 2 were quite demonstrative. This allowed evaluation of "detectability" of the test.

Figure 2: Arithmetic CFT titres obtained with twofold dilutions of a highly positive serum.



Most laboratories succeeded in getting very straight curves (02, 03, 04, Cirad). Curves for lab 01 and 05 were less straight which could be an indication of pipetting repeatability problems. However globally all the curves are sufficiently linear to let the laboratories pass the test.
Concerning the titres almost all laboratories obtained titres that did not differ from more than one dilution. The exception is laboratory 05 that obtained lower titres. The highest detectability was obtained by laboratory 03.

The results obtained by the other two groups of sera more or less confirmed these first results (Figure 3)

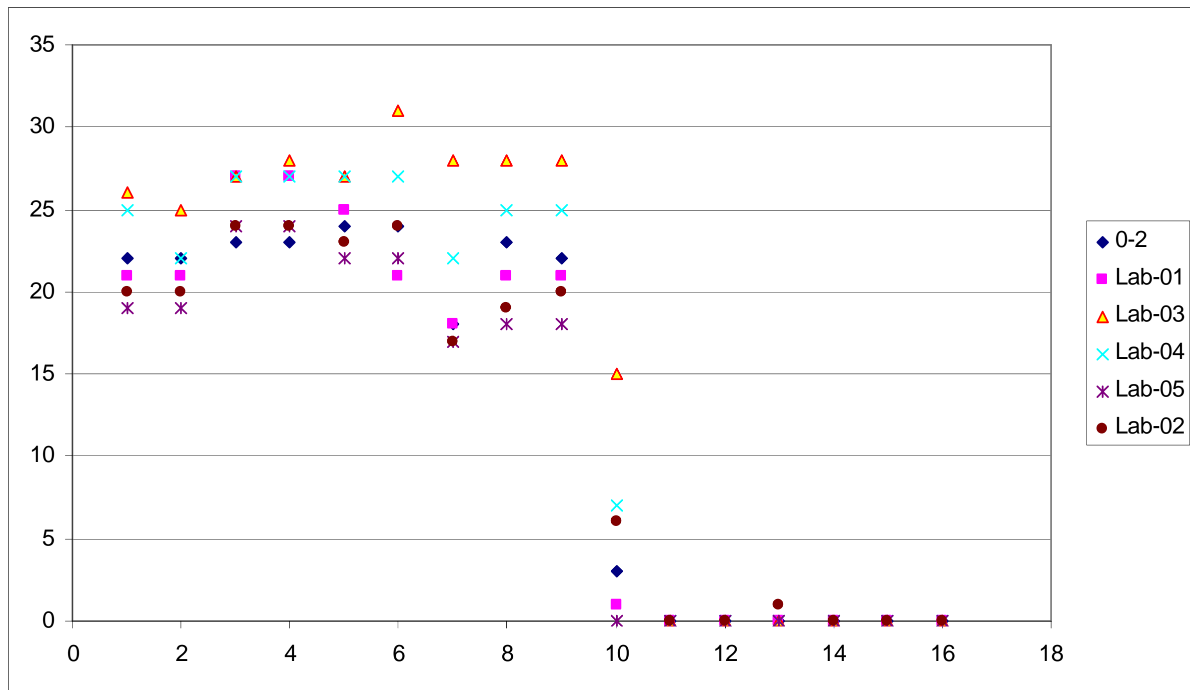


Figure 3: CFT titres obtained with the positive and negative groups of sera

Nine positive sera were found positive by all laboratories and the 6 negative sera (of French origin) were also found negative by all laboratories. A single serum yielded different results (serum N°10). This serum comes from a French cattle that had been immunized repeatedly with killed MmmSC antigen and adjuvant. At Cirad this serum gives low CFT titres but very high cELISA results. In this interlaboratory trial this serum was found negative by some laboratories, dubious by others, like Cirad, and positive by others, including Laboratory 03 that had the highest titres with the positive serum in dilutions.
Again in that test it must be noted that a serum may have titres that vary quite a lot from one laboratory to another (more than 2 twofold dilutions). In the case of an outbreak confirmation this will have no importance as many sera will exhibit very high titres. Such variations may have more impact when performing sero-prevalence surveys. Slightly positive sera may be found positive by some and negative by others. This variation may even be greater when converting individual prevalence rates to “herd prevalence” rates as a single positive in a herd may turn this herd becoming positive. What remains to be seen is if an increase sensitivity of the CFT may not be correlated to a lower specificity. Our test did not indicate this as laboratory 03 which had the highest sensitivity did not fail the specificity test. However we had only very few negative samples and a specificity check must certainly be performed by each laboratory with a representative number of negative sera.
Finally the CFT results can be summarized as a table 6

CFT	Sensitivity	Specificity	Detectability
Lab-01	PASS	PASS	PASS
Lab-02	PASS	PASS	PASS
Lab-03	PASS	PASS	PASS
Lab-04	PASS	PASS	PASS
Lab-05	PASS	PASS	Fail
Lab-06	Fail	Fail	Fail

Table 6: Final results for the complement fixation test

NB: Lab-06 failed because incomplete results were obtained at the time this report was prepared.

3.5.2 Serology, competition ELISA (cELISA)

Four laboratories sent cELISA results. Out of them one (Lab-07) was not in a position to receive sera of African origin at the beginning of this ILAT. In replacement it received two sets of a highly positive serum in dilution (French cattle immunized with a killed antigen). For this laboratory results were analyzed mostly for detectability and reproducibility. Finally Lab-07 received the same sera as the others at the beginning of December but results were not available when writing this report.

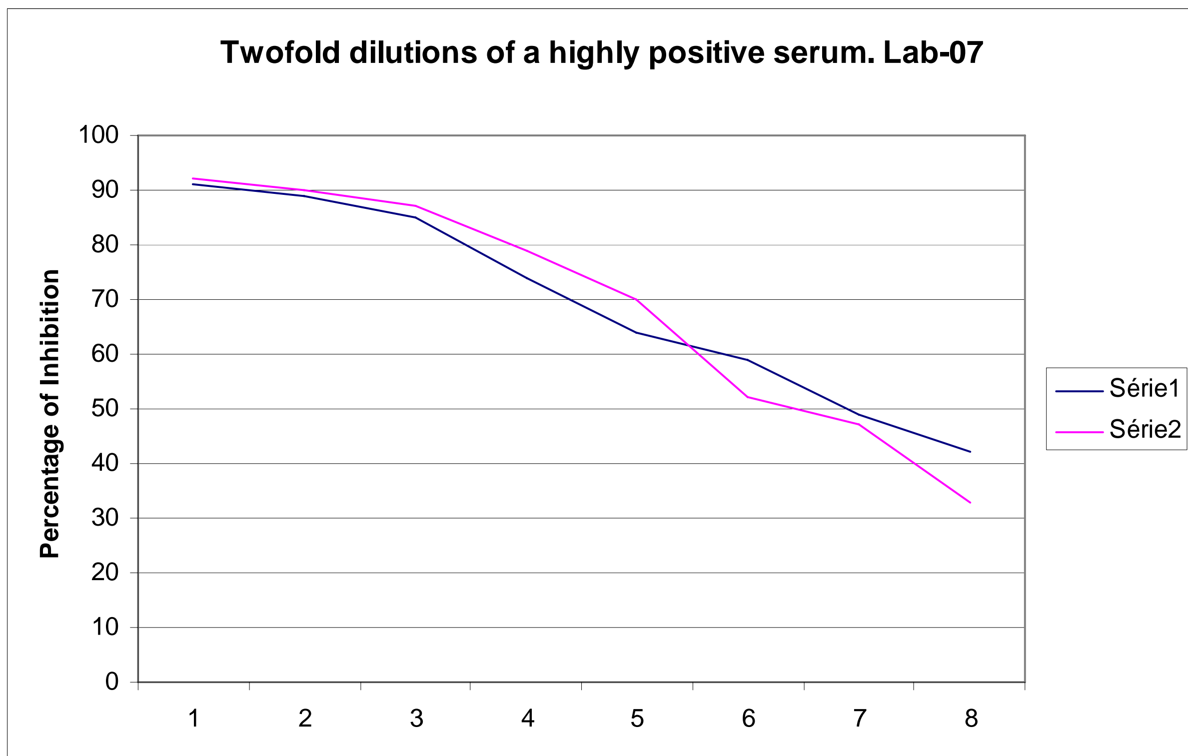


Figure 4: cELISA resultzs of Lab-07 with a known high positive serum in dilutions.

Lab-07 passes the test for detectability and reproducibility. The curves obtained with the duplicated samples are strictly strait and parallel (Fig 4). Furthermore, all duplicated sera had titres that did not differ from more than 10% which indicated that the test was highly reproducible in that laboratory.

Results for lab-06 were too irregular for being incorporated in a graph. One explanation could be a mis-identification of the samples in the plate.

Results with the common set of serum samples and for the other laboratories are presented in figure 5. Regularity of pipetting was good for all these laboratories as the curves are very straight. Three results were included for CIRAD (0-1, 0-2, 0-3) as they represent three sets of results obtained by three different technicians as an "abilitation exercise".
Lab-02 is the only one to have some detectability problems as the curve is shifted towards lower values. This may be a minor problem linked to the performing of the test or the way

values are calculated. The same set of sera is to be tested again and discussions are underway to try and identify the cause of this shift.

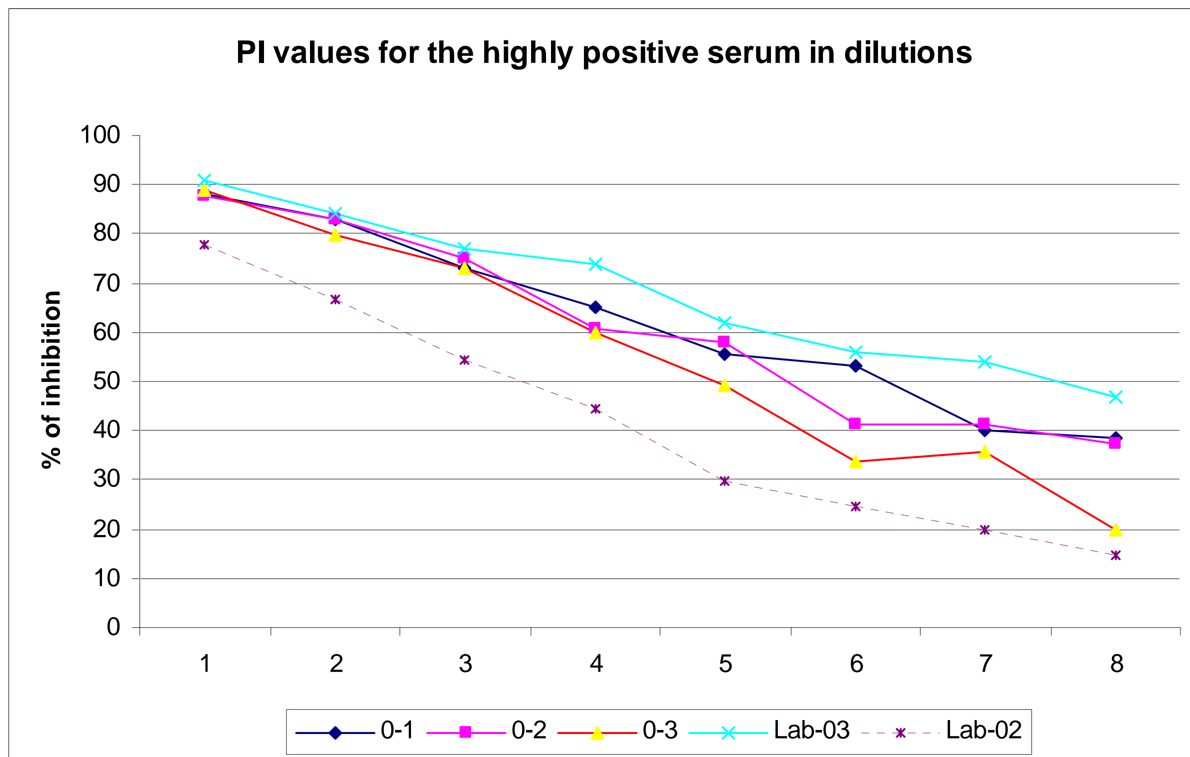


Figure 5: cELISA values obtained with a known highly positive serum in dilutions.

The set of positive and negative sera gave very similar results between CIRAD and lab-03 (Fig 6). All positive sera were found positive and all sera from French origin were found negative. Furthermore, duplicated sera gave very similar results.
Again Lab-02 had lower values than the other laboratories. This happened already with the set of diluted positive serum and it is an indication that there must be a unique factor at stake. Once it is found it should not be difficult for Lab-02 to find similar values as the others.

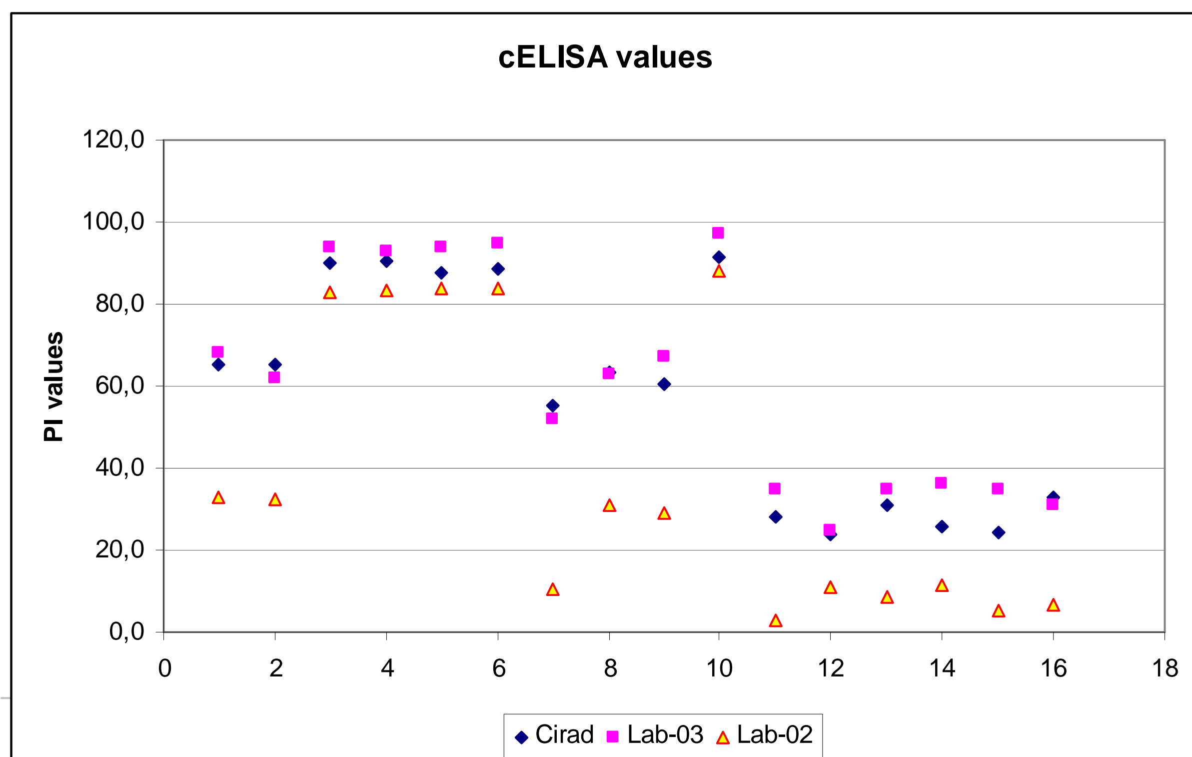


Figure 6: cELISA results obtained with the positive and negative groups of sera.

The final result for the cELISA can be summarized with a table (Tab 7)

cELISA	Specificity	Sensitivity	Detectability	Repeatability
Lab-02	PASS	fail	fail	PASS
Lab-03	PASS	PASS	PASS	PASS
Lab-06	fail	fail	fail	fail
Lab-07	PASS	NA	PASS	PASS

NA: Not Applicable

Table 7: Final results for the cELISA test
NA: Not applicable

3.5.3 Serology, Immunoblotting (IBT)

Results by IBT are more difficult to analyze because of the low number of laboratories that took part to this test and because the two that sent results did not test the same set of sera at first. However a number of results can be presented.
Lab-07 tested first a series of twofold dilutions of a positive serum of an immunized cattle of French origin. As this serum was also tested in cELISA the two techniques can be compared in terms of sensitivity. Obviously the two techniques seem to have a very similar sensitivity as the tests become negative at the same dilution. (1/32 to 1/64) (Table 8 right part)
Lab-04 that tested the common set of sera found some discrepant results between CFT and IBT. Some of the sera yielded negative results by IBT as they were positive by the CFT. However, the volume of serum was apparently barely sufficient to perform the test according to Lab-04 SOP. Additional volumes of serum were then sent to Lab-04 to confirm these results. Upon retesting, two known positive sera were again found negative by IBT with the absence of the 48KDa band. In that case there was no doubt on the positivity of the sera as they came from an experimental infection.

The common set of sera is still to be analyzed by two other laboratories and comparisons of results obtained in the three laboratories will be much more informative. The absence of a band could be explained by a variability of response in the infected animals and this would sign a true lack of sensitivity of this test. Other possibilities exist as the absence of the band could be explained by the time when the serum was collected especially if the animal was at early stage of infection, as IBT detects mainly IgGs. In fact there is no single test capable of detecting all animals in all stages of the disease. This is why some countries chose to perform two different tests and especially CFT and IBT. The presence in the sera of antibodies against the 5 specific antigens indicates an on-going infection.
The IBT has a sensitivity of 87.5% in the detection of infected animals at the individual level, but sensitivity rises to 92.5% in the detection of infected herds, allowing the definition of the sanitary state of the herd

Finally, at the last moment, Lab-07 was able to test the same batch of sera as Lab-04 (Table 8 left part). This laboratory found positive all the sera from the experimental infection, including those that had been found negative by lab-04. This tends to indicate a lack of sensitivity for lab-04 rather than a problem due to the time of serum collection. However lab-07 found negative some dilutions of the highly positive serum. The bands corresponding to these sera were notably paler than the others and this could explain a lack of detectability.

Table 8: Comparison of IBT results with titres obtained in CFT (Lab-04) or cELISA (Lab-07)

	Lab-04		Lab-07	Lab-07	
	CFT arth	IBT	IBT	cELISA	IBT
Positive serum	29	Pos	POS	91	POS
Positive serum 1/2	21	Pos	POS	92	POS
Positive serum 1/4	18	Pos	POS	89	POS
Positive serum 1/8	14	Pos	POS	90	POS
Positive serum 1/16	9	Pos	N	85	POS
Positive serum 1/32	7	Pos	N	87	POS
Positive serum 1/64	2	neg	N	74	POS
Positive serum 1/128	0	neg	POS	79	POS
	25	Pos	POS	64	POS
	22	Pos	POS	70	POS
	27	Pos	POS	59	POS
	27	Pos	POS	52	neg
	27	neg	POS	49	neg
	27	Pos	POS	47	neg
	22	Pos	POS	42	neg
	25	neg	POS	33	neg
	25	neg	POS	43	neg
	7	neg	POS	93	POS
	0	neg	Neg	24	neg
	0	neg	Neg	26	neg
	0	neg	Neg	12	neg
	0	neg	Neg	27	neg
	0	neg	Neg	0	neg
	0	neg	Neg	24	neg

4. CONCLUSION

First of all I wish to thank all participating laboratories. To my knowledge it was the first ring trial for CBPP organized jointly for European and African laboratories. Notwithstanding the actual results in the various tests, participating to a ring trial is a first step for evaluating the lab's performance and this ring trial can now be considered as a reference for any future similar exercise.

In some laboratories there is an obvious need to improve pipetting accuracy. This may be the easiest and most urgent action to be taken. Pipetting inaccuracy results could originate from four points: the pipette, the fitness of the tips with the pipette, the technician himself or the ELISA reader. Each of these points can easily be tested by repeating the test in various combinations. This is quite inexpensive as only the reference dye is needed and CIRAD can provide it at very low cost. Improvement in the serological tests can only be implemented after the laboratory (and the technicians) pass the test for pipetting accuracy.

A limited number of laboratories took part of the test for culturing and identification of mycoplasma cultures. For the SADC region this is certainly the weakest point. Cultivation of MmmSC is not particularly difficult and it can be done with ready-made media (hence solving the problem of quality management of home-made media). It seems then that solving this weakness is only a matter of will and investment. This problem could be solved easily with a limited amount of funds. Isolation of MmmSC strains is particularly important in the perspective of an eradication campaign and in the context of antibiotic use. Isolating MmmSC

strains will then enable testing their antibio-sensitivity and allow their molecular characterization.

Strangely enough, more laboratories performed PCR as compared to isolation. In most cases the results were correct and this is satisfactory as PCR can be used to rapidly identify CBPP outbreaks in supposedly free areas. PCR can also be used to monitor the onset of post-vaccinal reactions. However PCR is always prone to possible contamination problems and laboratories are encouraged to perform real-time PCR in the near future when a number of techniques are available in the literature.

CFT was the most widely used serological test in this trial. This is no surprise as this is the most ancient reference test of the OIE and it has been used for decades in Africa. Most laboratories passed the test except one (but results are still pending). Another one had a slight problem of detectability. It would have been interesting to get some pipetting accuracy result for this laboratory to possibly identify the origin of the problem.

cELISA was used by fewer laboratories. In two laboratories the results were strictly similar to Cirad's results although these laboratories were not familiar with the test. Furthermore, comparisons with IBT results showed that the two tests had similar sensitivities. Owing to the relative complexity of IBT this tends to prove that cELISA could be advantageous to use.

IBT was used by two laboratories but in different samples. Some sera from infected animals giving CFT positive result were not confirmed by IBT in one laboratory. The fact that the animal might be in an initial stage of infection, when all the antibodies against the 5 specific antigens where not yet present could have explained this discrepancy of results. However when a second laboratory performed the IBT on the same set of sera, all sera from the infected animals were found positive. This signs a certain lack of sensitivity for one laboratory. Globally this interlaboratory trial confirmed that IBT is a good test for the definition of the sanitary state of a herd. It may not be a gold standard for individual detection and it certainly needs some skill to perform it routinely with good repeatability.

Finally it must be stressed that rapid confirmation of CBPP outbreak or estimation of prevalence rates or losses due to CBPP will always result from comprehensive approaches including various information and test results. In that respect it is particularly important to ensure that the laboratory results are accurate. Quality assurance is the ultimate way to ensure this accuracy and it is to be hoped that more and more laboratories, within SADC and elsewhere will embark in this system. Let me hope that this interlaboratory trial has been of some help and that within a reasonable time some actions have been taken to correct the possible defects that have been identified.

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Annex 1. Transformation of classical CFT results into an arithmetic scale to ensure statistical analysis.

Dilution	Haemolysis inhibition	Titre
	-	0
1/10	+	1
	++	2
	+++	3
	++++	4
1/20	+	5
	++	6
	+++	7
	++++	8
1/40	+	9
	++	10
	+++	11
	++++	12
1/80	+	13
	++	14
	+++	15
	++++	16
1/160	+	17
	++	18
	+++	19
	++++	20
1/320	+	21
	++	22
	+++	23
	++++	24
1/640	+	25
	++	26
	+++	27
	++++	28
1/1280	+	29
	++	30
	+++	31
	++++	32
1/2560	+	33
	++	34
	+++	35
	++++	36

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