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# SADC REGIONAL INTER-LABORATORY PROFICIENCY TESTING EXERCISE FOR RABIES DIAGNOSIS

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29 NOVEMBER 2011



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Exercise funded by the OIE World Animal Health and Welfare Fund, with donations from the US Centres for Disease prevention and Control (CDC)

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## Acronyms

ABLV	Australian bat lyssavirus
ANSES	Agence Nationale pour la Sécurité Sanitaire [France]
ARC	Agricultural Research Council [South Africa]
CDC	Centres for Disease prevention and Control [US]
DRC	Democratic Republic of Congo
DUVV	Duvenhage virus
FAO	Food and Agricultural Organization of the United Nations
FAT	Fluorescent antibody test
FITC	Fluorescein isothiocyanate
FN	False negative
FP	False positive
GT	genotype
ISO	International Standards Organisation
IU	International unit(s)
LBV	Lagos bat virus
MOKV	Mokola virus
OIE	World Organisation for Animal Health
OVI	Onderstepoort Veterinary Institute [ARC]
PBS	Phosphate buffered saline
PT	Proficiency test(ing)
RABV	(classical) rabies virus
RNP	Ribo-nucleoprotein
SADC	Southern African Development Community
SEARG	Southern and Eastern Africa Rabies Group
SOP	Standard Operating Procedure(s)
SRR	Sub-Regional Representation [OIE]
SRR-SA	SRR for Southern Africa
UP	University of Pretoria [South Africa]
WHO	World Health Organisation



*This report can be downloaded from the OIE Africa website at :  
[www.rr-africa.oie.int/docspdf/en/2011/rabiespt.pdf](http://www.rr-africa.oie.int/docspdf/en/2011/rabiespt.pdf)*

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*All pictures courtesy of [www.worldrabiesday.org](http://www.worldrabiesday.org) (2011) unless mentioned otherwise.*

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### Useful web links

ANSES	<a href="http://www.anses.fr">www.anses.fr</a>
ARC	<a href="http://www.arc.agric.za">www.arc.agric.za</a>
CDC	<a href="http://www.cdc.gov">www.cdc.gov</a>
FAO	<a href="http://www.fao.org">www.fao.org</a>
Global Alliance for Rabies Control	<a href="http://www.rabiescontrol.net">www.rabiescontrol.net</a>
OIE Africa portal	<a href="http://www.rr-africa.oie.int">www.rr-africa.oie.int</a>
OIE rabies portal	<a href="http://www.oie.int/en/animal-health-in-the-world/rabies-portal">www.oie.int/en/animal-health-in-the-world/rabies-portal</a>
OIE	<a href="http://www.oie.int">www.oie.int</a>
OVI	<a href="http://www.arc.agric.za/home.asp?pid=373">http://www.arc.agric.za/home.asp?pid=373</a>
SADC	<a href="http://www.sadc.int">www.sadc.int</a>
SEARG	<a href="http://www.searg.info">www.searg.info</a>
UP	<a href="http://www.up.ac.za">www.up.ac.za</a>
World Rabies Day	<a href="http://www.worldrabiesday.org">www.worldrabiesday.org</a>

## BACKGROUND

As a direct result of the recommendations emanating from the bi-annual conference of the *Southern and Eastern African Rabies Group* (SEARG) which took place in Maputo (Mozambique, January 25-27, 2011), participants (including public health officers, animal health officers, researchers, vaccine producing organisations and medical practitioners) resolved that laboratory capacity for animal rabies in the SADC region should be enhanced. One of the ways to achieve this was to conduct a proficiency testing, whereby the *Onderstepoort Veterinary Institute* would send a blind panel of samples to other veterinary diagnostic laboratories in the SADC region in order to evaluate the competency of diagnosticians in these laboratories. In performing this exercise, laboratories would use a standardised *standard operating procedure* (SOP) and biologicals including the anti-rabies conjugate. This exercise would also verify whether the rabies diagnostic training by the *Onderstepoort Veterinary Institute* from July 27-31, 2009 had been attained.

## OBJECTIVES

- To prepare documents (acknowledgement forms, report forms), send calls for participation and request for import permits,
- To procure mice and materials for courier of samples,
- To select, prepare panel samples and test their stability,
- To courier panels of samples to national reference laboratories in the SADC region,
- To analyse and report the test results,
- To communicate to Heads of Laboratories, Chair SADC Laboratory Subcommittee and funders (OIE and FAO) and
- To prepare the proficiency test report to be submitted to the OIE SRR after completion of the training.

## DESIGN

### **Materials and methods:**

The intent to participate in the PT exercise and procedure to be followed was presented by Dr Sabeta at the SADC diagnostic sub-committee meeting held at the Kopanong Hotel and Conference Centre, Gauteng, South Africa (14-16 March 2011). Heads of Laboratories were contacted and requested to submit relevant documents for the importation of panels of samples and the anti-lyssavirus biological conjugate. Each laboratory was then assigned a random code to preserve anonymity. The panel of 11 samples to be tested (together with a vial of anti-rabies conjugate and instructions for reconstituting the conjugate), acknowledgement form (for checking the condition of the samples on receipt, Appendix 1) and the result form for each participating laboratory were sent to Heads of Laboratories (Table 1). Panels of samples were sent to all the laboratories, with the exception of Angola, Madagascar, Mauritius and the Seychelles (who did not indicate their intent to participate).

**Table 1:** List of SADC countries invited to participate in the proficiency test exercise.

Country number	Country	Code	Contact person
1	Angola	Lab 01	
2	Botswana	Lab 02	Dr L Modisa
3	Democratic Republic of Congo	Lab 03	Dr L Mulumba
4	Lesotho	Lab 04	Dr C Kubi
5	Madagascar	Lab 05	
6	Malawi	Lab 06	Dr G Njunga
7	Mauritius	Lab 07	Dr AK Srivastava
8	Mozambique	Lab 08	Dr Sara Acha
9	Namibia	Lab 09	Ms Marais/ Dr S Khaiseb
10	Seychelles	Lab 10	Dr A Leon
11	South Africa (Allerton)	Lab 11	Ms J Le Roux
12	South Africa (OVI)	Lab 12	Dr W Shumba
13	Swaziland	Lab 13	Dr M Dube
14	Tanzania	Lab 14	Dr C Ngeleja
15	Zambia	Lab 15	Dr G Monga
16	Zimbabwe	Lab 16	Dr P Makaya

## COMPOSITION OF THE PANEL

The samples of this panel were composed of freeze-dried and homogenised mouse-infected brain materials, all prepared at Onderstepoort and original brain tissues submitted for routine rabies diagnosis (Table 2). All were positive for rabies virus antigen by the FAT method. The order of the samples in Table 2 is similar to that in the form for the inter-laboratory results filled by individual laboratories. The panel of samples included panel 1 with Lagos bat virus (LBV, genotype 2), Mokola virus (MOKV, genotype 3), Duvenhage virus (DUVV, genotype 4), mongoose rabies virus (genotype 1) and a negative brain tissue derived from a bovine. Panel 2 consisted of 5 positive samples, 2 strong positives and three samples diluted with uninfected brain tissues (1:5; 1:100 and 1:400) and a negative sample.

**Table 2:** Panel of samples sent to participating laboratories in SADC member countries.

Panel 1			
Virus material	Laboratory number	Code	Comments (if any)
Lagos bat virus	RA390	201105PT294	
Mokola virus	173/06	201105PT148	
Duvenhage virus	SA/06	201105PT280	
Mongoose rabies virus	1164/10	201105PT136	
Bovine (negative)	366/11	201105PT154	
Panel 2			
Virus material	Laboratory number	Code	Comments (if any)
Positive A	344/11	201105PT115	Field strain (of bovine origin, mongoose strain)
Positive B	341/11	201105PT272	Field strain (dog strain)
Positive C (1:5)	343/11	201105PT209	Diluted (Dog strain)
Positive D (1:400)	173/06	201105PT178	Diluted (MOKV)
Positive (1:100)	351/11	201105PT157	Diluted (Dog strain)
Negative	367/11	201105PT139	Canine origin

In order to validate the effect of transportation on the suitability of samples, a panel of 11 samples was sent to Dr Joule Kangumba (Potchefstroom Veterinary Laboratory, North West Province, South Africa) by courier on the 6 of June 2011 and sent back to Onderstepoort (received a week later). The panel was retested on the 14 of June and expected results were obtained indicating that the transportation had no effect on the condition of samples and did not interfere with the test. The panel of samples was then sent to all the Heads of Laboratories on the 20th of June, with the exception of the consignment for the DRC which was sent later on the 22nd of June, 2011. The shipment of panels of samples was done at ambient temperature by an international courier company. In the letter that accompanied the samples, laboratories were advised to store the panel at 4°C until analysis. The 22 of July was set as the deadline for the return of the test results with the exception for the DRC (29th July).

### **Some definitions used on the interpretation of the results**

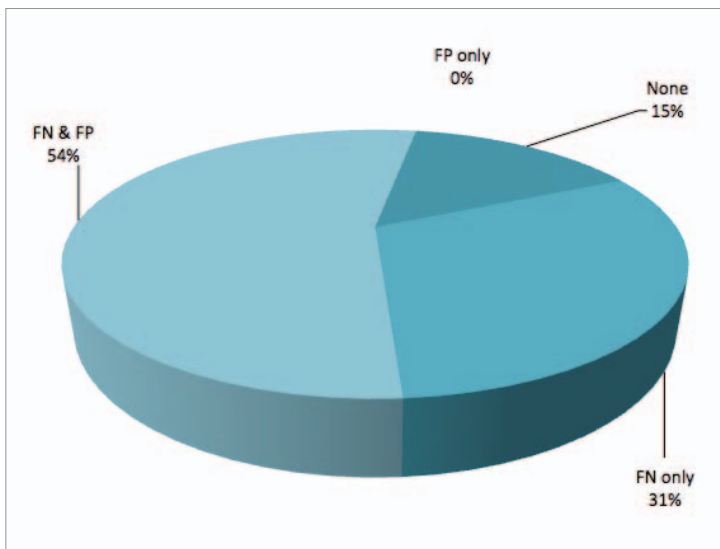
- A discrepancy - this is when a result given by a laboratory is different from the expected results (positive or negative). Discrepancies include false positives (FP) [the test result should actually be negative] and false negative (FN) results [the test result should actually be positive].
- Sensitivity =  $\frac{\text{Number of true positives found by laboratories}}{\text{Total number of positive samples (true pos + false neg)}} \times 100$
- Specificity =  $\frac{\text{Number of true negatives found by laboratories}}{\text{Total number of positive samples (true neg + false pos)}} \times 100$
- The terms “sensitivity” and “specificity” are used only for the purpose of evaluating the performance of laboratories in the frame of the inter-laboratory trial. Therefore the sensitivity and specificity of this inter-laboratory trial cannot be compared to the classical sensitivity and specificity of a technique as this is calculated on the basis of a random sampling.

**NB:**

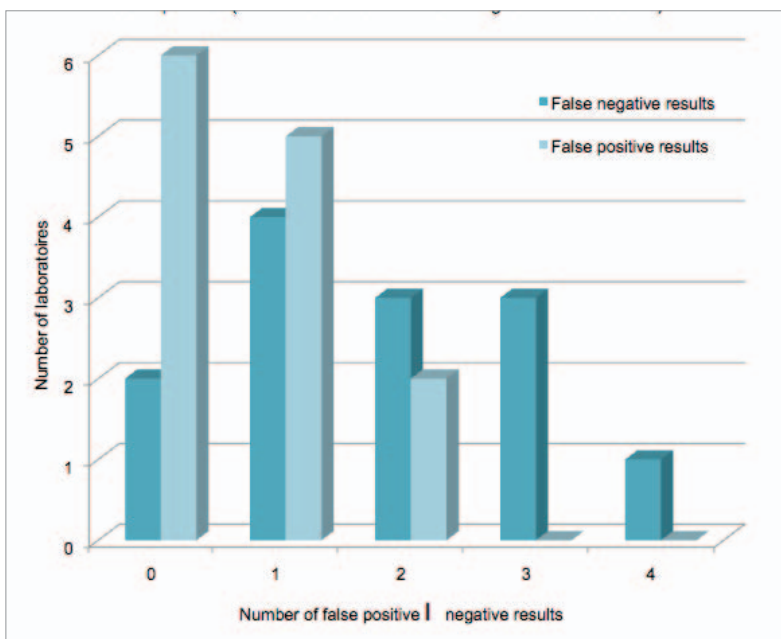
For this proficiency test, a specificity and sensitivity of 65.4% and 80% respectively were obtained. This means the laboratories that participated in this proficiency test exercise are at different levels of competence. In future and in the subsequent PT exercises, all participating laboratories should therefore aim to improve on this. The specificity and sensitivity of the actual test (FAT) is approximately 100% and between 99.2% and 99.9%.

**RESULTS**

Most of the laboratories produced satisfactory results. Collectively, the performance of the laboratories can be significantly improved by reducing the number of false negative (n=23) and false positive results (n=9) which impact on the sensitivity and specificity respectively. The sample that was diluted 1:400 was the least detected and this possibly indicates the low sensitivity of some microscopes (may have a bearing on the service history and status of this very important piece of equipment) or could be the inexperience of some readers. This is given that all laboratories were provided with the same biological conjugate. The false positive results obtained by some laboratories for a negative control are a concern though. One possible reason for this result is that some laboratories may not have included Evans Blue in the test procedure as indicated in the protocol. In international interlaboratory proficiency testing exercise, co-ordinated by the French Agency for Food Environmental and Occupational Health and Safety (ANSES, France) in 2010, five laboratories returned three false positive results [4.6% of negative samples] and seven false negative results [8% of positive samples] (Robardet *et al.*, 2011).



Results of the blind panel exercise for the 13 participating laboratories: discrepancies (in percentage of laboratories)



Results of the blind panel exercise for the 13 participating laboratories: discrepancies (in number of laboratories having 0 to 4 false results)



## DISCUSSION AND RECOMMENDATIONS

This is the first coordinated exercise done by the OIE Rabies Reference Laboratory (ARC-OVI) for SADC MS. In future, it is anticipated that larger amounts of sample will be provided per vial. This is so because in the case of some freeze-dried mouse brain materials samples thin suspensions were obtained after re-suspending in water, making it very difficult to make a good brain smear on the slides. Note: it would have been appropriate to assess the efficiency of transportation of the panels of samples to each respective laboratory. The dates reflected on some of the returns do not show exactly when the panels were received. It should be emphasised that in future laboratories should return the acknowledgement form as soon as they have received the panel.

**Based on this preliminary report**, it is recommended that:

1. A follow up questionnaire to be sent to all participating laboratories with a view to establish how the whole exercise can be improved.
2. Specific attention and visits be undertaken particularly to those laboratories that may require technical assistance.
3. An assessment for the quality management system be undertaken in all laboratories participating in the PT exercise (by the Quality Management Committee).
4. Proficiency test exercises for rabies should be continued.
5. Funding for these PT exercises be provided by regional and international organisations such as the SADC, OIE and FAO.
6. The use of good negative and positive controls is recommended (Dean *et al.*, 1996) because it can reduce the number of false positives.

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Dean, D., Abelseth, M.K., Atanasiu, P., 1996. The fluorescent antibody test, In: Meslin, F.X., Kaplan, M.M., Kprowski, H (eds). *Laboratory Techniques in Rabies*. World Health Organisation, Geneva, pp. 88-95.

OIE, 2008. *Manual of Diagnostic Tests and Vaccines for terrestrial Animals (mammals, birds and bees)*, 6th edition, 598p.

Robardet, E., Picard-Meyer, E., Andrieu, S., Servat, A., Cliquet, F., 2011. International interlaboratory trials on rabies diagnosis: an overview of the results and variation in reference diagnosis techniques (fluorescent antibody test, rabies tissue infection test, mouse inoculation test) and molecular biology techniques. *Journal of Virological Methods*. 177: 15-25.

## APPENDIX 1 : GENERIC LETTER

You will find enclosed the test panel and all the required documentation for you to perform the inter-laboratory test for rabies for 2011:

The test panel consists of two parts: panel A (5 coded samples) and panel B (5 coded samples). Each sample contains 1 ml of homogenate of brain tissue and is susceptible to infection by classical rabies virus (RABV), Lagos bat virus (LBV), Mokola virus (MOKV) and Duvenhage (DUVV) strains. For panel A only, please indicate the order of fluorescence as instructed in the results form.

- Please return the acknowledgement form on the receipt of the panel of samples
- A result form is attached for the FAT
- Your laboratory code is .

The panel of samples should be kept refrigerated (at 4°C) until the analysis is undertaken. Each vial should be reconstituted with 1 ml of distilled water. As soon as the samples have been reconstituted, please immediately proceed with the test. In the test, you should include your usual controls in the procedure to validate the test. We expect all results to be returned by the end of July 2011. All results will be blindly analysed and a report will be sent to each of the participants.

Please do not hesitate to contact us should you have any question or clarification. We thank you in advance for participating in this proficiency test.

1b Acknowledgement form: attached

1c Results form: attached

1d Individual laboratory results are also attached.

1e Standard operating procedure used in the PT exercise.

A handwritten signature in black ink, appearing to read 'P. Sabatini', is located in the lower-left quadrant of the page.

First report: 20 October 2011

Revised version: 29 November 2011

## APPENDIX 2 : RESULTS


Inter-laboratory test for the fluorescent antibody test (FAT) for SADC member countries for 2011

### Sample of a country - Results

Laboratory code: (Not disclosed)

Date of results: (Not disclosed)

	Tube code	Expected	Obtained result	Order of increasing fluorescence (for panel 2 only)
Panel 1	126	-	-	Bovine negative
	208	+	-	Mongoose rabies virus
	226	+	+	Duvenhage virus
	265	+	+	Lagos Bat Virus
	276	+	+	Mokola virus
Panel 2	127	+	+	1:100 dilution dog strain
	157	+	-	Mongoose strain positive A (bovine origin)
	175	+	+	Dog strain positive B
	206	-	+	Canine negative
	232	+	+	1: 5 dilution dog strain positive C
	263	+	-	1: 400 Mokola strain

Signature 

## *SADC Harmonized SOP of the Rabies Fluorescent Antibody Test (FAT)*

Edition 1.0

Date Issued: 1 October 2010

Withdrawal Date:

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## INTRODUCTION

Rabies is a fatal viral zoonotic disease of significant public and veterinary health threat. The causative agent of the disease, rabies virus (RABV), mainly infects warm-blooded vertebrates (both mammals and humans). The RABV infection is generally transmitted by the bite of infected animals, most commonly dogs and other wildlife carnivore species.

Rabies and rabies-related viruses are highly neurotropic members of the Lyssavirus genus, family Rhabdoviridae. Within this family of viruses are seven genotypes (gts) (1-7), namely:- classical rabies virus (gt1), Lagos bat virus (LBV, gt2), Mokola virus (MOKV, gt3), Duvenhage virus (DUVV, gt4), European bat lyssavirus type-1 and 2 (gts 5 & 6 respectively) and the Australian bat lyssavirus (ABLV, gt7) as well as unassigned viruses. Genotypes 1-4 have been identified from canid and chiropteran species in southern Africa, notably Zimbabwe and South Africa. In these countries where there is a large biological diversity of lyssaviruses, it is recommended to use polyclonal rabies conjugate for diagnosis. Natural infections with gts 2, 3 and 4 viruses have only been encountered on the African continent. With the exception of MOKV, all the lyssavirus genotypes and putative genotypes have been isolated exclusively from Chiropteran species.

Virus factories in infected cells appear as inclusion bodies, consisting predominantly of viral capsids (Ribo-Nucleo-Protein, RNP). The histologically demonstrable rabies inclusion bodies are called "Negri bodies". Among the laboratory tests available for routine diagnosis, the fluorescent antibody test (FAT), which detects viral antigen is the gold standard and should be used by all laboratories performing such work. The test is fast (results are obtained in less than 3 hours), comparatively inexpensive and more accurate (can detect 97-99% of positive specimens) than either histological based or mouse inoculation tests. FAT is the recommended by both the WHO and the OIE. Negative samples with human contact (bite) history should be subjected to viral propagation techniques (mice or cell cultures) and subsequently confirmed by FAT.

### **1. PURPOSE, SCOPE AND PRINCIPLE OF TEST**

The purpose of this SOP is to describe the method of rapid diagnosis of rabies by FAT on fresh or glycerol-preserved brain specimens. In brief, this method is used for detecting rabies inclusion bodies (viral antigen) in cells of acetone-fixed brain smears. The smears are incubated with FITC-labelled anti lyssavirus polyclonal antibody. Unbound antibody is then removed by washing and smears are examined by fluorescence microscopy. In rabies positive specimens the antibodies bind to the antigen and produce apple-green fluorescing inclusion bodies or viral aggregates when viewed under a fluorescent microscope.

### **2. SAFETY CONSIDERATIONS AND PRECAUTIONS**

#### **2.1. Personnel**

2.1.1. All laboratory personnel who handle and work with suspected rabies virus infected tissue specimens must be well trained, competent and comply with national biocontainment and biosafety regulations to protect staff from contact with pathogens. The conditions should include:

2.1.2. All personnel involved in rabies testing should receive pre-exposure immunisation.

2.1.3. Only personnel who demonstrate an antibody titre of 0.5 international units (IU) per ml or higher should be allowed to handle the suspected rabies infected specimens.

2.1.4. Personnel should be routinely monitored every 6 months for adequate rabies neutralising antibodies. Booster vaccinations must be given when the titre falls below 0.5 IU/ml. In the

absence of serological monitoring, the vaccination regimen should consist of a booster vaccination at 1 year and thereafter every 1-3 years, depending on the vaccine.

2.1.5. Appropriate protective clothing must be worn at all times.

#### **2.2. The laboratory operations**

2.2.1. The laboratory should comply with the OIE guidelines on Biosafety and biosecurity in the veterinary microbiological laboratory and animal facilities. Such practices include that:

- The specimens must only be processed in a Class II Biological Safety Cabinet.
- Aerosols – high speed centrifugation and any procedure that generates aerosols should be carried out in tightly closed containers and possibly under a negative draught hood.
- All contaminated instruments and utensils must be sterilised by autoclaving immediately after the test procedure is finished and before they are washed.
- Disposable items must be placed into sterilising bags and must be sterilised by autoclaving before disposal.
- Disinfection of biological safety cabinets and used instruments should be done with disinfectants such as 1% Virkon and/or other virucidals efficient to kill lyssaviruses.

### **3. SPECIMEN REGISTRATION**

- 3.1. Allocate rabies laboratory numbers using the ISO country code (2 characters), laboratory code and number representing the total number of samples received to date.
- 3.2. Assign to each specimen a rabies laboratory number, starting with the ISO country code, followed by the specimen number e.g. 01 if it is the first sample of the year, followed by the last 2 digits of the year e.g. a specimen from Swaziland will be registered as: SZ01/10 or if there are two labs in that country then it would be SZCVL01/10 or SZM01/10, if it is from Mbabane Veterinary Laboratory.
- 3.3. Record this rabies laboratory number on the submission form (Appendix 1) as well as the specimen bottle.

### **4. REQUIRED MATERIALS**

#### **4.1. Equipment and Facilities**

- 4.1.1. Fluorescent microscope, Zeiss or equivalent, with mercury vapour lamp, 50 or 100 watt (450-490 nm excitation filters, and 510 nm stop filter).
- 4.1.2. Refrigerator (+2 to +8°C).
- 4.1.3. Freezers, chest or upright, with temperature of not less than -20°C (e.g. -70°C).
- 4.1.4. Incubator, temperature capability of 37°C±2°C).
- 4.1.5. Double door autoclave, any brand.
- 4.1.6. Class II biological safety cabinets, any approved brand.
- 4.1.7. Single channel Micropipettor for drawing 100µl volumes.
- 4.1.8. Single channel Micropipettor for drawing 0.5-10µl volumes.
- 4.1.9. Vortex mixer.
- 4.1.10. pH meter.
- 4.1.11. Dark room.
- 4.1.12. Forceps.
- 4.1.13. Scissors.
- 4.1.14. Standard laboratory timers.

#### **4.2. Other supplies**

- 4.2.1. Closed plastic container for use as a humidified chamber
- 4.2.2. Microscope slides, clear, frosted one end, any brand, non-fluorescent
- 4.2.3. Cover slips 13 mm thick, any brand
- 4.2.4. Petri dishes, disposable or other suitable container to place brain material
- 4.2.5. Non fluffing high quality absorbent paper towel
- 4.2.6. Coplin staining jar
- 4.2.7. HB pencil for labelling slides
- 4.2.8. Lens cleaning tissue
- 4.2.9. Squeeze/wash bottle with PBS
- 4.2.10. A suitable containers with virucidal disinfectant capable of killing rabies virus
- 4.2.11. PPE including Nitrile/Latex powder free disposable Gloves
- 4.2.12. Disposable tips (1-10 µl, 20-200 µl,)
- 4.2.13. Autoclavable bags

#### **4.3. Reagents**

- 4.3.1. 0.01M Phosphate buffered saline (PBS), pH 7.2-7.4 (Refer to Appendix 2 for preparation instructions) or tablets/sachets.
- 4.3.2. Heat sterilized distilled or deionized water, or water of an equivalent purity.
- 4.3.3. High-grade (99.9 to 100%) acetone (CH<sub>3</sub>)<sub>2</sub>CO (MW 58.8), Assay by (GC) is minimum of 99.5%, ASC grade, or similar. Verify quality of acetone before use (Refer to instructions under quality control).
- 4.3.4. High grade glycerol
- 4.3.5. Anti-lyssavirus FITC polyclonal conjugate (can be obtained from the ARC-Onderstepoort Veterinary Institute ([www.arc.agric.za](http://www.arc.agric.za)), SANOFI Pasteur ([www.sanofipasteur.com](http://www.sanofipasteur.com)), Chemicon ([www.chemicon.com](http://www.chemicon.com)) with a predetermined working dilution.
- 4.3.6. Lens cleaning fluid (Appendix 5).
- 4.3.7. Mounting media/mountant (Appendix 4).
- 4.3.8. Positive and negative control brain specimens.
- 4.3.9. Fresh or 50% glycerol saline (Appendix 3) preserved test brain specimen.
- 4.3.10. Evans blue. Counterstain added to the working dilution of the conjugate is optional. Evans Blue counterstain (0.5%) can be aliquoted and stored at +4°C for up to 6 months and indefinitely at -20°C. The amount of counterstain added to the conjugate is determined by titration when the working dilution of the conjugate is determined. Due to counterstain, the cells will be noticeably red, but should not be strongly red as to diminish the specific green fluorescence. An Evans Blue concentration of 0.00125% works very well in many laboratories. This concentration is prepared by adding 2.5 µl of 0.5% stock dye solution per ml of conjugate diluent.



## **5. TEST PROCEDURE**

### **5.1. Preparation for the test**

#### *5.1.1. Personnel qualifications and training:*

- Personnel must be competent in the following:
  - a. Preparation and proper handling of test reagents and biological materials.
  - b. Calibration, maintenance and use of instruments listed in section 4.
  - c. Adherence to good laboratory practices.

#### *5.1.2. Preparation of equipment/instrumentation*

- Equipment is calibrated and certified according to respective testing Laboratory Standard Operating Procedures.
- Use clean and or sterile equipment
- Clean the objectives of the microscope lens with lens cleaning fluid (see appendix 5 on how to prepare).

#### *5.1.3. Preparation of reagents, test specimens, controls and other materials*

- Prepare the diluents for the conjugate which is 0.01M PBS pH 7.2±0.2 with 0.5% Evans blue. This can be prepared and aliquoted into 1 ml volumes at -20°C .
- Determine the optimum working dilution of the anti-lyssavirus FITC polyclonal conjugate as per manufacturer's instructions to provide an excellent staining of viral antigen in brain smears. On the day of specimen testing, prepare only enough of the optimum working dilution conjugate for the number of samples to be tested by adding working dilution of the conjugate into the diluent above (PBS with 0.5% Evans Blue).
- On each day of performing a test, ensure that there is sufficiently chilled acetone preferably kept -20°C.
- Keep microscope slides in an acetone bath at room temperature for degreasing and take them out when needed for staining.
- Dry the slides using appropriate absorbent paper towel.
- Phosphate buffered saline (PBS) 0.01M pH 7.2-7.4 (Refer to Appendix 2 for instructions on how to prepare the buffer).
- Keep fresh filtered acetone in a Coplin or staining jar in a freezer (-20°C).
- Preserve fresh brain specimens that cannot be tested on the day of arrival in 50% glycerol-saline (refer to Appendix 3 for instructions to prepare glycerol-saline). Specimens in 50% glycerol-saline can be stored at room temperature for a maximum of 2 weeks, or frozen at -20°C or at a lower temperature overnight.
- Thaw frozen control samples on the day of testing by placing them in a biosafety cabinet at room temperature for 30-60 minutes.
- Prepare mounting media (Refer to Appendix 4 for instructions on how to prepare the mounting media).

### **5.2. Sample preparation**

*ALL PROCEDURES SHOULD BE PERFORMED IN A CLASS II BIOLOGICAL SAFETY CABINET AND GLOVES AND A GOWN MUST BE WORN AT ALL TIMES.*

*NOTE 1: PREFERABLY, SUBMITTED SPECIMENS NEED TO BE TESTED ON THE DAY OF ARRIVAL AND IF THIS IS NOT POSSIBLE, SHOULD BE TESTED AT THE EARLIEST CONVENIENCE. USE A FRESH SET OF STERILE, CLEAN EQUIPMENT AND SUPPLIES FOR EACH BRAIN SPECIMEN (FORCEPS, SCISSORS, GLASS SLIDE ETC).*

*NOTE 2: ALWAYS PREPARE THE CONTROL SLIDES LAST.*

5.2.1. Remove clean slides from acetone and dry them with appropriate absorbent paper.

5.2.2. Label the frosted end of the slide with the corresponding rabies specimen identity using a pencil.

5.2.3. Using forceps, take out the brain specimen from its original container and place on a petri dish or any other suitable shallow container while exposing the different parts of the brain required for the test.

5.2.4. Using a pair of scissors, cut small samples (about 2 to 3 mm) of the fresh brain specimen from the brain stem (thalamus, pons, medulla oblongata), cerebellum and hippocampus and place each piece onto two ends of the microscope slide/ wooden tongue depressor to constitute a composite of samples in a pair. The composite samples should be placed within a close distance so that when an impression smear is prepared all the brain samples in the composite are exposed and the resulting smear is about 1cm in diameter. In case the brain specimen is preserved in 50% glycerol saline, each cut brain sample should be blotted on paper towel to remove excess glycerol and then treated as fresh ones.

- 5.2.5. Using the forceps, return the original brain specimen into its original container and place it at -20°C or lower temperature for further storage.
- 5.2.6. Make impression smears by lightly pressing the microscope slide for staining on the two sets of brain composite samples.
- 5.2.7. Prepare the negative and positive controls in the same manner as test samples and include them in every staining.
- 5.2.8. Air dry smears for five minutes at room temperature.
- 5.2.9. Discard all the contaminated equipment/materials used to obtain the smear into a container with disinfectant.

### **5.3. Fixation**

- 5.3.1. Immerse the slides into fresh cold acetone and then keep them at -20°C for 20 minutes.
- 5.3.2. Air-dry the fixed slides for approximately 5 minutes in the Class II Biological Safety cabinet until all traces of acetone and moisture have evaporated.

### **5.4. Staining procedure**

- 5.4.1. Prepare a sufficient amount of anti-lyssavirus FITC conjugate according to conjugate dilution titration table.
- 5.4.2. Place the slides into a humidified chamber. The humidified chamber is prepared by placing absorbent paper in an enclosed container with a flat bottom, and then pouring some PBS on the paper to create moisture during the incubation step. The paper should not be too wet as this may interfere with conjugate.
- 5.4.3. Apply freshly-prepared working dilution conjugate to cover the smear (approximately 150 µl per smear will suffice for a 1 cm diameter of smear. Too little conjugate will result in excessive drying while too much of the conjugate will increase the possibility of the conjugate running off the slides and these could both produce undesirable results).
- 5.4.4. Place lid on humidified chamber.
- 5.4.5. Incubate the slides in the humidified chamber at 37°C for 30 minutes.
- 5.4.6. At the end of the 30-min incubation, remove excess conjugate and wash or rinse the slides in PBS three times and then blot dry them on paper. Please ensure that the slides are not completely dry!

### **5.5. Mounting**

- 5.5.1. Place one drop of mounting media on the slide and place a coverslip on top, ensuring that the mounting fluid is evenly distributed over the smear, while minimising air bubbles on the smear.
- 5.5.2. Place slides in covered slide container and take to microscope room (Dark room) for reading.

## **6. READING AND RECORDING OF RESULTS**

- 6.1. Two people must read all the stained slides and control smears. Positive controls must be positive and the negative control sample must be negative for the results to be acceptable.
- 6.2. The stained slides should be examined using the 40X objective starting with positive and negative controls. In positive controls, the presence of rabies antigen is demonstrated by appearance of apple green oval to round shaped inclusion bodies of various sizes, some as dust particles studded against a black to dark greenish background, while such fluorescing inclusion bodies should not appear in negative control smears.
- 6.3. Continue reading the test smears.
- 6.4. Grade the smear quantitatively with a score of +1 to +4, depending on the abundance of viral antigens and give a quantitative grade of +1 to +4 depending on the intensity of fluorescence and record the results in the appropriate test results recording system.

## **7. WASTE DISPOSAL**

All the generated waste should be sterilised in autoclave before disposal according to the waste disposal SOP of each respective laboratory.

## **8. QUALITY CONTROL**

- 8.1. Each new lot of acetone must be checked to ensure that the acetone does not interfere with the staining of the brain smears.
  - 8.1.1. Select tissue from 4 to 6 previous specimens that have stained both weakly and strongly positive for rabies virus.
  - 8.1.2. Prepare two slides from each specimen.
  - 8.1.3. Fix one set of slides in the acetone in use and the other set in the new lot of acetone.
  - 8.1.4. Follow the procedures for fixing and staining.
  - 8.1.5. Read both sets of slides noting the amount of virus as well as intensity of staining.
  - 8.1.6. Both sets of slides must have identical results. If the new acetone does not meet this criterion, discard and purchase another lot.

7.2 Similarly, the optimal working dilution of a new batch of conjugate must be determined by titration. Naturally, the working dilution may differ between laboratories depending on the microscope optimal system.

7.2.1 Select known positive and negative brain material to prepare controls.

Inoculate suckling mice with a 10% suspension of sample that previously tested positive with a score of +4. Pool brain samples from which succumb and test positive for rabies virus antigen. To prepare the negative control, inoculate suckling mice with PBS. Harvest brain tissues after 28 days, pool and place in vials.

7.2.2 Prepare two-fold dilutions of the conjugate in PBS starting from 1:80 to 1:2560.

7.2.3 Prepare two slides for each specimen.

7.2.4 Fix the slides as described in the protocol.

7.2.5 Follow the procedures for fixing and staining.

7.2.6 Read both sets of slides noting the amount of virus and intensity.

7.2.7 The least conjugate dilution that gives an excellent staining will be used as a working dilution of that batch.

7.2.8 Prevent conjugate contamination by dispensing 1-ml quantities of the diluted conjugate into eppendorf tubes and store at -20oC or lower.

**9.** External quality control will involve an annual participation by each of the SADC member laboratories in an FAT proficiency testing on a panel of rabies positive and negative samples.

## **10. REFERENCES**

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## 11. APPENDICES

### APPENDIX 1: ISO country codes and submission form ISO country codes

Country	Code
Angola	AO
Botswana	BW
Lesotho	LS
Democratic Republic of Congo	CD
Madagascar	MG
Malawi	MW
Mauritius	MU
Mozambique	MZ
Namibia	NA
Seychelles	SE
South Africa	ZA
Swaziland	SZ
Tanzania	TZ
Zambia	ZM
Zimbabwe	ZW

### SADC RABIES SPECIMEN SUBMISSION FORM Namibia Central Veterinary laboratory

Please note that specimens in formalin cannot be reliably tested for rabies. Brain samples for Rabies must be submitted fresh on ice or in Glycerolsaline.

Rabies Number: e.g NA01/10	SPECIES	SENDER'S REFERENCE
Date sent:		GVO/Technician:
Date received:		Tel No.
Government Vet area		District
Sender		Province
Owner's name , address and contact number		
GRID REFERENCE:		

#### HISTORY

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No of Human Contacts	Bitten	<input type="checkbox"/>	Handling/Saliva/Scratch	<input type="checkbox"/>
Manner of Death	Killed	<input type="checkbox"/>	Died Naturally	<input type="checkbox"/>

Specimen quality	
Preservative	

#### For Official use only

FAT result	
Further tests and Results:	
Comments:	

## APPENDIX 2.

### *Preparation of 0.01M PBS, pH 7.2-7.4*

Sodium chloride (NaCl)	100 g
Potassium chloride (KCl)	2.5 g
Potassium dihydrogen orthophosphate (KH <sub>2</sub> PO <sub>4</sub> )	2.5 g
Disodium hydrogen phosphate dodecahydrate (Na <sub>2</sub> HPO <sub>4</sub> .12H <sub>2</sub> O)	14.5 g

Add 10 litres of distilled water and dissolve completely. The PBS should have a pH of between 7.2 and 7.4. Do not use PBS which has some precipitates.

## APPENDIX 3.

### *Preparation of 50% glycerol saline preservative solution*

Double-distilled glycerol	500 ml
Phosphate buffered saline (PBS) pH 7.4	500 ml
Merthiolate (not necessary)	0.1 g

## APPENDIX 4.

### *Preparation of mounting media for FAT: buffered glycerin solution pH 8.5*

#### *A. Concentrated solutions*

Solution A. pH 4.2	
KH <sub>2</sub> PO <sub>4</sub>	89.82 g
NaCl	8.5 g
Deionised water	1 litre
Solution B pH 8.8	
KH <sub>2</sub> PO <sub>4</sub>	114.96 g
NaCl	8.5 g
Deionised water	1 litre

#### *B. Buffer preparation*

Solution A	1 ml
Solution B	100 ml

#### *C. Mounting medium*

Bidistilled glycerol	9 vol
Buffer	1 vol

Adjust the pH to 8.5 with solution A or B. Mounting solution should be kept at room temperature and a new batch is prepared every 3 months.

## APPENDIX 5.

### *Preparation of lens cleaning fluid*

Isopropyl alcohol	50 ml
Distilled or deionised water	150 ml
Teepol	3 drops



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